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THE DETERMINATION OF THE GASES OF THE BLOOD.

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CONTENTS.

During several years of use certain improvements have developed in the technique for using the apparatus designed by one of us for extracting from the blood and measuring the carbon dioxide (Van Slyke, 1917), oxygen (Van Slyke, 1918), and carbon monoxide (Van Slyke and Salvesen, 1919). A technique has furthermore been developed whereby all the blood gases may be determined in one sample of 1 cc. of blood. The purpose of the present paper is to present these developments.

A Modified Form of Blood Gas Apparatus for Especially Accurate Work.[1]

For analyses in which it is desirable to reduce the error in reading the gas volumes to less than 0.005 cc. an apparatus modified in one respect from the original macro form (Van Slyke, 1917) is now used. The upper, graduated stem of the apparatus has been reduced in bore from 4 mm. to 2.7 mm., so that its

[1] The apparatus, with the water jacket and mechanical shaking device, may be obtained from Emil Greiner, 55 Fulton Street, New York.

cross-section is halved. Consequently the length of tube holding 1 cc. of gas is lengthened from 75 to 80 mm. to 150 to 160 mm. and it is divided into 100 instead of 50 divisions. In the finer tube gas volumes can be estimated to 0.1 of a division, or 0.001 cc. Also, because of its narrowness the column of liquid can be easily seen through and the meniscus read sharply, even when the solution is deeply colored, as in whole blood analyses.

The only difference in manipulation necessitated by the change in construction is in the manner in which the apparatus is shaken in extracting the gases. The extraction is accomplished by whirling the blood mixture about the wall of the chamber, rather than by the repeated inversion of the apparatus. The measuring tube is of such small diameter that liquid caused to lodge in it by inverting the apparatus is dislodged with some difficulty. The mechanical shaker described by Stadie (1921) is well adapted for use with this form of the apparatus.

In the narrow tube CO_2 and O_2 may be absorbed by admission of alkali and pyrogallol solutions as readily as in the wider tube of the original apparatus. It is desirable, however, in admitting the absorbing solution from the cup at the top of the apparatus to open the cock so slightly that the solution trickles down only one side of the narrow measuring tube below. If the solution enters so rapidly that it forms a solid column in the measuring tube it is dislodged by tapping the tube with the finger, or by running in mercury from the cup. When a viscous absorbing solution, like pyrogallol, is used, it is followed by a little water to clear the inner wall of the measuring tube. Even after careful admission of water or absorbing solution it is the rule for a slight but measurable amount to stick in the upper end of the measuring capillary, just below the stop-cock. A few gentle taps with the finger, or the admission of a drop of mercury from above, suffice for dislodgment.

After the admission of any fluid from the cup into the chamber, time should be allowed for drainage before the gas volume is read. A few seconds suffice if only 0.1 cc. of gas remains to be measured, but 2 minutes are required if the volume approaches 1 cc.

In order to obtain the benefit of the accuracy obtainable with the fine bore apparatus, it is necessary to use it in a room in which the temperature does not vary by more than 1°C., or to provide

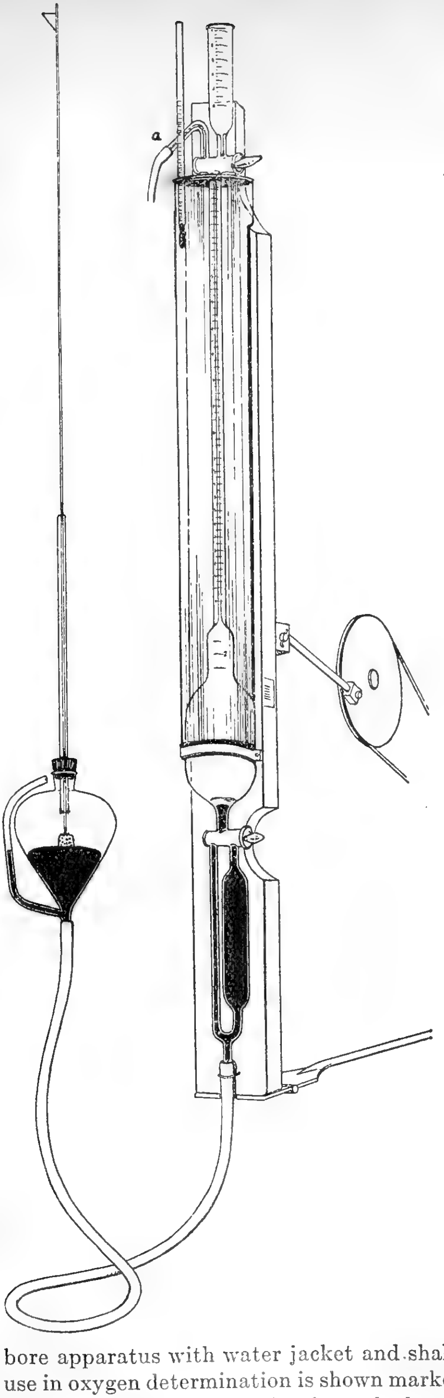

Fig. 1. Fine bore apparatus with water jacket and shaking device. A Stadie scale for use in oxygen determination is shown marked on the side of the supporting board. The stick projecting from the levelling bulb is for measuring 500 mm. reduction in pressure. It is well to insert a glass air trap between the apparatus and levelling bulb, as described for the micro-apparatus (Van Slyke, 1917, p. 363).

it with a water jacket such as is shown in Fig. 1. The apparatus may be shaken by hand, or may be attached to a hinged board, as shown in Fig. 1, so arranged that the shaking may be done by a motor.

The manipulation of the apparatus with the finer bore requires slightly more time and care than that of the original apparatus, and the latter is adequate for most purposes, such as the determination of the oxygen capacity of blood as a measure of the hemoglobin, and of the carbon dioxide as a measure of the alkaline reserve, both values being capable of determination by the original apparatus to within 1 per cent of the amounts normally measured. For some purposes, however, such as the determination of all the gases in 1 cc. of blood, the determination of carbon monoxide, or of the slight amount of nitrogen gas in blood, or when it is essential to keep the error in CO_2 determinations below 0.5 volume per cent, or in oxygen determinations below 0.25, the fine bore apparatus is desirable.

In calibrating the apparatus, we attach to the bottom, either by sealing or by joining glass on glass with a heavy-walled piece of suction tubing, a glass tube bearing a fine bore stop-cock and drawn out into a fine capillary. Through the latter water is drawn up into the apparatus by means of suction applied to the outlet above the upper cock. When the entire apparatus and the upper outlet are filled with water, the cock of the capillary attached below is closed, and the suction is discontinued. The upper cock of the apparatus is then turned, so that the chamber is connected with the empty cup. The water is now delivered through the cock of the attached capillary, 0.1 cc. at a time. The drops are caught in a weighing bottle containing a layer several mm. thick of paraffin oil. After the delivery of each drop, the tip of the capillary is touched to the surface of the oil, to detach all adhering water except a uniform minimum. The drops of water sink beneath the oil, and loss of weight by evaporation is prevented with completeness that is not obtainable by merely using a covered weighing bottle, which must be opened to receive each addition of water. The weighings are made to the nearest milligram.

The calibration may also be performed with mercury, as described for the micro-apparatus.[2] In this case sealing the

[2] Van Slyke (1917), p. 365.

delivery capillary to the apparatus is necessary, as the pressure is sufficient to expand appreciably a rubber tube.

The constancy of results which may be obtained with the fine bore apparatus is indicated by Table I, which shows the duplicate readings obtained in a series of analyses performed by Dr. J. P. Peters, Jr., in determining the CO_2 absorption curve of a specimen of blood.

TABLE I.

Results of Series of Duplicate Determinations of CO_2 in Whole Blood with the Fine Bore Apparatus.

1 cc. of blood was used for each analysis.

No.	V. Total volume of gas extracted.	$V - CO_2$. Observed volume after absorbing CO_2 with NaOH.	CO_2 By difference.
	cc.	*cc.*	*cc.*
1	0.735	0.042	0.693
	0.725	0.033	0.692
2	0.604	0.130	0.474
	0.603	0.130	0.472
3	0.704	0.128	0.576
	0.696	0.122	0.574
4	0.763	0.126	0.637
	0.764	0.126	0.638

Magnification of Small Gas Volumes by Reduction of Pressure.

When an accurate measurement of very small gas volumes (*e.g.*, less than 0.05 cc.) is desired, as may be the case in determining the N_2 or CO content of blood, it is desirable for measurement to increase the gas volume by reducing the pressure. The reduction is accomplished by holding the levelling bulb lower, by the desired distance, than it would be placed in order to put the gas in the apparatus under atmospheric pressure. We have found 500 mm. of mercury to be a convenient reduction in pressure for this purpose. For readily locating this level we have used the light rod with the marker attached near its top, shown projecting up out of the levelling bulb in Fig. 1. The rod is a pine stick 2.5 mm. in diameter and a little over 500 mm. long.

The marker is a piece of stiff paper glued on 501 mm. from the bottom. The stick passes down into the levelling bulb through a piece of glass tubing about 10 cm. long, which is held upright in a rubber stopper and serves to keep the stick in a vertical position during readings. At its lower end, within the levelling bulb, the stick terminates in a cork, which floats on the mercury. It dips 1 mm. below the surface in the case of our apparatus (the exact distance would, of course, vary for different sticks and corks) and to correct for this depth of immersion the distance from the bottom of the cork to the marker is made 501 instead of 500 mm.

The measurement is made merely by placing the bottom of the marker at the level, in relation to the mercury and water levels inside the apparatus, at which one would place the level of the mercury itself in the bulb in order to obtain atmospheric pressure within the apparatus.

The volume of gas read is reduced to atmospheric pressure by multiplying by the factor $\frac{B - H - w}{B - w}$, B being the atmospheric pressure, H the height from the mercury in the levelling bulb to the marker on the stick (*e.g.*, 500 mm.), and w the vapor tension of the water at the temperature at which the reading is taken.

For example, a gas volume is read as 0.133 cc. at 25°, and 765 − 500 = 265 mm. pressure. The vapor tension of water at 25° is 23.6 mm. Hence, the volume of gas measured moist at room temperature and pressure would be $0.133 \times \frac{265 - 23.6}{765 - 23.6} =$ 0.0433. This is reduced to 0°, 760 mm. measured dry by the usual factor given in Table XIII, which in this case is $0.888 \times \frac{765}{760} = 0.894$. The consequent volume at 0°, 760 mm. is therefore $0.0433 \times 0.894 = 0.0387$ cc.

The Nitrogen Gas Content of Blood, and its Effect on the Calculation of Results of Other Blood Gases.

In the original paper on the determination of oxygen, the oxygen and nitrogen were extracted from the blood and measured together, and the oxygen was estimated by deducting the

relatively small amount of nitrogen, which was calculated from Bohr's determinations (1905) of the solubilities of gases in blood and water. The solubility coefficient of nitrogen gas in water (the volume of gas reduced to 0°, 760 mm., which 1 volume of water dissolves when in equilibrium with the gas at 760 mm. tension) at 38° is 0.0122; in blood Bohr found the solubility coefficients of chemically inert gases to be 92 per cent as great as in water. The volume of nitrogen calculated to be held in solution by 100 cc. of water at body temperature and 760 mm. barometric pressure (diminished by 48 mm. vapor tension of water) in equilibrium with the atmosphere is, therefore, $100 \times 0.0122 \times 0.791 \times \frac{760-48}{760} = 0.90$ cc. For blood, the calculated amount would be 0.92 as great, or 0.83 cc.

Earlier analysts had found as much as 4 to 5 volumes per cent of nitrogen gas in the blood, and attributed the excess above that which water would dissolve as due to leakage of air into the evacuated apparatus used.

Bohr and Henriques (1897) by more accurate methods determined the nitrogen gas, along with the carbon dioxide and oxygen, in both arterial and venous bloods of a number of dogs (twenty-two analyses). They found that the nitrogen contents varied from the theoretically expected 0.8 volume per cent to 1.7 volumes per cent, the average and most of the individual figures being in the neighborhood of 1.2 volumes per cent. In a paper published at nearly the same time Bohr (1897) found that when shaken with air at room temperature blood of the ox and dog, and likewise 10 to 12 per cent solutions of oxyhemoglobin, absorbed 1.65 to 1.98 volumes per cent of nitrogen. Water under the same conditions absorbed only 1.30 volumes per cent. Peculiarly enough, oxygen-free nitrogen was absorbed in no greater proportion by blood than by water. For the fact that blood in contact with air absorbs more nitrogen than does water, there appears to be as yet no well grounded explanation, although Bohr suggested that some easily dissociated oxide of nitrogen might be formed when nitrogen and oxygen meet in the presence of dissolved hemoglobin. In Bohr's later paper (1905) on the absorption coefficients of gases in blood and water the bearing of these results on the absorption coefficient of nitrogen in blood is not considered, and the coefficient is given as 92 per cent of that in water.

When the original paper on the determination of oxygen in the blood was published (Van Slyke, 1918) Bohr's earlier data (1897) were overlooked, and the corrections for nitrogen gas were based on the solubility coefficient given in his 1905 publication. Van Slyke and Salvesen (1919), in later analyses in which the oxygen and nitrogen were measured separately, the oxygen being absorbed in the apparatus by pyrogallol, found that the

nitrogen gas in rabbit blood approximated 1.2 volumes per cent, a figure which agrees with the average found in dog blood by Bohr and Henriques (1897). Smith, Dawson, and Cohen (1919–20) have recently reported further analyses showing a nitrogen content of blood higher than that estimated from the solubility coefficient.

We have performed a number of determinations of the nitrogen gas content of blood, both as drawn from the veins and after equilibration with air at room temperature. It was found that the same results were obtained when no reagents were added as when ferricyanide was used to release the oxygen, or acid to release carbon dioxide. Consequently in the series here reported the nitrogen was determined by extraction in a vacuum without the addition of reagents. The apparatus employed was the model with the finer bore measuring tube described at the beginning of this paper. The technique used was the following:

3 cc. of water and a few drops of octyl alcohol were freed completely from air by vacuum extraction in the apparatus in the usual manner. The extraction was always repeated in order to make certain that not even 0.001 cc. of air was left. 1 to 2 cc. of the water were run up into the cup, and 5 cc. of fresh blood, which had been drawn under paraffin oil without exposure to air, were run under the water and into the chamber of the apparatus. (The fact that the water during its brief contact with air in the cup absorbed no measurable amounts of air was proved by controls.) The blood-water mixture was evacuated in the apparatus and the gases were shaken out by hand for 3 minutes; longer shaking was found not to increase the yield of nitrogen gas. After release of the vacuum the oxygen and carbon dioxide were absorbed by allowing a few drops of alkaline pyrogallol solution to run slowly from the cup down the measuring tube. When absorption was complete, as shown by shrinkage of the gas to a constant volume, the somewhat viscous pyrogallol solution was washed from the inner walls of the tube by means of a little water, and the volume of unabsorbed gas was measured as nitrogen. The results are given in Table II.

The results in Table II confirm those of Bohr in showing that the blood, both in the veins and after aerating at room temperature, contains about 0.5 volume per cent more nitrogen gas than calculated from the solubility coefficient of the gas in water. Our results, perhaps because the simplicity of our method has reduced the chance of error, vary over a smaller range than Bohr's. In venous blood the total range is 1.36 ± 0.11 volumes per cent of

nitrogen, while in blood aerated at 23 – 30° *in vitro* it is 1.52 ± 0.2 volumes per cent.

The results in Table II, as well as those of Bohr above referred to, include as "nitrogen" all the gas extracted by evacuating blood, and left after absorption of oxygen and carbon dioxide. Regnard and Schlœsing (1897) found besides nitrogen 0.04 volume per cent of argon in this gas fraction from horse blood, 2.00 volumes per cent still remaining and being calculated as nitrogen. Whether it actually is all nitrogen, or a mixture of nitrogen and gases, such as methane, hydrogen, etc., not yet identified, is still uncertain. Hartridge (1919–20) has found as much as 1 volume per cent of carbon monoxide in the blood of a heavy

TABLE II.

Nitrogen Gas in Whole Blood.

No.	Subject.	Duplicates.		Mean.	Temperature.
Venous blood as drawn.					
		vol. per cent	*vol. per cent*	*vol. per cent*	°*C.*
1		1.36	1.42	1.39	
2	St.	1.30	1.39	1.35	
3	M. L.	1.63	1.30	1.47	
4	Kel.	1.33		1.33	
5	Schu.	1.36		1.36	
6	M. S.	1.30		1.30	
7	Koh.	1.43		1.43	
8	Dog 1.	1.44	1.26	1.35	
9	" 2.	1.31	1.40	1.36	
10	Dr. B.	1.25		1.25	
Average				1.36	
Venous blood saturated with air at room temperature.					
1	E.	1.78	1.63	1.71	24
2	St.	1.47	1.64	1.56	23
3	Schu.	1.30	1.31	1.31	29
4	Koh.	1.52		1.52	25
5	V. S.	1.60	1.60	1.60	23
6	Dog 2.	1.40	1.41	1.41	25
7	Ox.	1.55		1.55	30
Average				1.52	

tobacco smoker, but there appears to be no reason to believe that carbon monoxide forms any part of the unexplained "nitrogen" ordinarily present in normal blood. The probability that the residual gas is actually nitrogen is strengthened by the fact that it is increased by aerating the blood at room temperature. The reverse would be expected if any considerable proportion of the residual gas were other than nitrogen.

It is evident from the above that when the nitrogen and oxygen, or nitrogen and carbon monoxide, of the blood are measured together, the volume of the gas accompanying the nitrogen cannot be estimated by deducting the volume of nitrogen estimated (as 0.83 volume per cent of the blood) from its solubility coefficient, without introducing an error of approximately plus 0.5 volume per cent into the calculation of the oxygen or carbon monoxide.

There are two alternatives. (1) The average N_2 content of blood as empirically determined (Table II) may be subtracted from the $N_2 + O_2$ or from the $N_2 + CO$ in order to estimate the O_2 or the CO. (2) The gas measured with the nitrogen may be determined by absorption with a proper reagent (*e.g.*, pyrogallol for oxygen). The details of the calculations, from analyses made by both alternatives, of oxygen content, oxygen capacity, carbon monoxide content, and of methemoglobin, the determination of which is based on oxygen capacity determinations, will be taken up in the following pages in connection with the discussions of these determinations.

Determination of Oxygen.

1. Changes in Technique.

A. Reduction in Amount of Ferricyanide Used.—We have reduced the amount of potassium ferricyanide per cc. of blood from 60 or 70 mg. (0.4 cc. of saturated solution for 2 cc. of blood) to 10 mg., which, as shown in Table III, is still twice the minimum necessary amount. The ferricyanide solution used contains 20 gm. per 100 cc. Of this 0.1 cc., containing 20 mg. of the salt, is used for 2 cc. of blood.

The smaller amount of ferricyanide causes as rapid and complete an evolution of oxygen as do larger amounts, and it has two

advantages over the latter. First, the smaller amount does not appreciably retard laking, and it is consequently unnecessary to wait for laking to become complete in the apparatus before the ferricyanide is introduced. Also, the possibility of error from incomplete laking is avoided. Second, reduction in the amount of ferricyanide reduces the formerly somewhat annoying amount of precipitate formed by interaction with the mercury in the apparatus.

B. Use of Water Instead of Ammonia for Laking Blood.—In the original procedure (Van Slyke, 1918) the blood, before addition of ferricyanide, was laked in the apparatus with saponin in a dilute ammonia solution, the proportions of blood and ammonia

TABLE III.

Effect of Varying Amounts of Potassium Ferricyanide on the Volume of Oxygen Liberated from 2 Cc. of Normal Human Blood.

Ferricyanide added to 2 cc. of blood.	$O_2 + N_2$ measured at 20°, 765 mm.	Calculated O_2 content of blood.*
gm.	*cc.*	*vol. per cent*
0.005	0.41	17.3
0.010	0.53	22.8
0.020	0.53	22.8
0.100	0.53	22.8

* 1.36 volumes per cent of N_2 being subtracted from $O_2 + N_2$ after reduction of the total volume to 0°, 760 mm.

being those previously adopted by Haldane and by Barcroft in their well known methods for determination of blood oxygen.

We have found that the use of such a mixture may introduce two errors, both usually small but frequently measurable by our present technique: (1) the alkalinity of the mixture is not sufficient entirely to prevent in all cases the escape of CO_2, particularly when the room temperature is high, and (2) the alkaline reaction accelerates some oxidative process by which part of the oxygen freed is slowly consumed. Hence, as shown by Table V, when the oxygen is freed in too alkaline a solution, the entire amount is not obtained.

With the dilute ammonia formerly used, the slight alkalinity caused but little oxygen consumption, sometimes none (Table VI) and the average error from this source was approximately

balanced by the error in the opposite direction due to the small amount of CO_2 set free. Consequently the normal figure for the oxygen capacity of human blood determined by the former technique is not affected by present changes.

At a temperature of 20°, and with ammonia of at least 0.06 N concentration (obtained by diluting 1 cc. of ordinary concentrated ammonia to 200 cc.), the CO_2 tension is usually too near zero to affect measurably the volume of the extracted gases (no decrease observed when the gases are washed with NaOH solution). If the temperature rises to 25 or 30°, however, or if by reason of standing or preparation from a weak stock solution the ammonia is of less than usual concentration, enough CO_2 may escape with the O_2 to increase the calculated volume per cent of the latter by several tenths, and occasionally more. There is also a difference in bloods, some freeing measurable amounts of CO_2 under conditions where others do not. Presumably bloods richer in hemoglobin, which acts as a weak acid, require more alkali to reduce their CO_2 tension to zero.

The obvious way to prevent mixture of CO_2 with the $O_2 + N_2$ would be to increase the alkali added to the blood to such an extent that there would in all cases be a sufficient excess. Such addition, however, increases the error due to oxygen consumption.

The latter error, like that due to CO_2 escape, may be nil or slight within the 2 minutes that usually suffice to extract all the oxygen. It is not uncommon, however, on extracting for a third minute, to observe a decrease of a few thousandths of a cc., amounting to 0.1 to 0.3 volume per cent of the blood.

At present, therefore, we use water for laking the blood, which seems to obviate the error due to oxygen consumption. The 10 to 20 volumes per cent of CO_2 that accompany the oxygen are removed after the extraction by absorption with NaOH solution, before the $O_2 + N_2$ volume is measured. Consequently the danger of error from admixture of CO_2 with the $O_2 + N_2$ is also avoided.

The avoidance of ammonia has an additional advantage in reducing the insoluble black precipitate which forms in its presence by the interaction of ferricyanide and mercury. With water as diluent and the lesser amounts of ferricyanide now used the precipitate is reduced to practically nil.

C. Details of Present Oxygen Determination.—The apparatus is washed out twice before each analysis with water, in the rapid manner described later in connection with the CO_2 determination (p. 22), in order to remove the alkali used to absorb CO_2 in any previous analysis. For 2 cc. of blood 6 cc. of water, 0.3 cc. of 1 per cent saponin (Merck) solution, and 2 or 3 drops of caprylic alcohol are introduced into the apparatus and freed of air, as described in the original paper (Van Slyke, 1918), the extraction being repeated until no more air is obtained.

Nearly the entire 6 cc. is then forced up into the cup of the apparatus. The blood is stirred to assure even distribution of corpuscles, and drawn into a pipette calibrated to *deliver* 2 cc. between two marks, of which the lower is 3 or 4 cm. from the tip. The pipette is introduced under the water solution in the cup, so that the tip rests on the bottom near the capillary. As the blood flows out of the pipette held in the left hand, the stopcock is partially opened with the right, so that the blood accompanied by some of the water, flows at once on into the chamber of the apparatus. The layer of blood need never rise more than 1 or 2 mm. above the bottom of the cup, and the slight amount adhering is washed completely into the chamber by the water which follows after all the blood has been delivered.

Before the last cc. of water is readmitted, 0.10 to 0.12 cc. of a solution, containing 20 gm. of potassium ferricyanide per 100 cc., is added and thereby introduced into the chamber after the blood. (The ferricyanide may be measured with sufficient accuracy as 3 drops from a dropper which delivers 1 cc. in 25 to 30 drops.) A mercury seal is made, and the apparatus is evacuated and shaken for 3 minutes. Usually 2 minutes, frequently less, are sufficient, but repeated trials have shown that 3 minutes are sometimes required before the last traces of oxygen are extracted. Owing to the continued slow evolution of CO_2 from the unalkalinized mixture, it is not possible to check the completeness of the $O_2 + N_2$ extraction by repeated measurements of the $O_2 + N_2 + CO_2$ volume; so it is desirable to continue the extraction in each case for a sufficient period to insure its completion. Extraction being complete, the vacuum is released by opening the lower cock, and the mercury together with the reaction mixture is allowed to run up into the chamber of the

apparatus. The gases obtained from 2 cc. of blood are normally about 0.50 cc. of O_2, 0.015 cc. of N_2, and 0.2 cc. of CO_2, about one-fifth of the total blood CO_2 being evolved under the conditions of the analysis. In order to absorb the CO_2 the levelling bulb is placed at such a height that the mercury in it is slightly below the level of the mercury in the apparatus, so that a slight negative pressure is maintained in the latter. 0.5 cc. of 0.5 N NaOH solution, previously saturated with air or oxygen, is admitted from the cup of the apparatus and allowed to trickle slowly down the inner wall to absorb the CO_2 from the gas mixture. If, as is frequently the case with the fine bore apparatus, the latter part of the solution enters as a solid column instead of running down the walls, it is dislodged by letting a little mercury pass down through it in a fine stream from the cup above. Since the mercury droplets are coated with alkali solution, they rapidly complete the absorption of CO_2 as they pass the gas column.

Even when all the alkali solution appears to flow down the sides of the gas pipette a little usually remains just below the stop-cock. If neglected, this amount introduces an error of from 0.005 to 0.010 cc. in the gas volume reading, but it can be easily dislodged by a drop of mercury run in as above described.

After absorption of CO_2 is complete time for drainage of the alkali solution must be allowed before the reading is taken (see p. 2).

For the reading the height of the levelling bulb is regulated by the scale described by Stadie (1921) in the accompanying paper, and shown in Fig. 1 of this paper. The use of the scale may be obviated by trapping the extracted solution in the bulb below the lower cock, as in the determination of CO_2 (Van Slyke, 1917), before the vacuum is released.

2. *Calculations Connected with Oxygen Determinations.*

A. Calculation of Total Oxygen Content of Blood.—The total oxygen content of blood includes both the oxygen chemically bound by hemoglobin and that held in physical solution by the blood fluids.

When the oxygen and nitrogen are extracted and measured together, as described in the original paper, the calculation is made as there

described, except that instead of subtracting the theoretically estimated 0.9 volume per cent of nitrogen from the $O_2 + N_2$ content the empirically determined *1.36 volumes per cent are subtracted. When from the mixture of $O_2 + N_2$ the O_2 is directly determined by absorption* with pyrogallol, as described by Van Slyke and Salvesen (1919), the empirical correction for nitrogen is, of course, avoided. Since absorption is completed in 1 minute it adds but little to the time required for an analysis. It is a useful check whenever there is reason to suspect leakage of air into the apparatus, as such an occurrence greatly increases the residual nitrogen gas.

B. Calculation of Oxygen Combined with Hemoglobin in Blood as Drawn.—The gases obtained together as described by extraction of arterial or venous blood collected out of contact with air are: (1) oxygen chemically combined with hemoglobin, (2) oxygen physically dissolved in the blood, and (3) nitrogen physically dissolved. For dissolved nitrogen the value as empirically determined above is 1.36 cc. per 100 cc. of blood. For oxygen the volume physically dissolved depends upon the partial pressure of oxygen with which the blood is in equilibrium. Arterial blood normally is in approximate though not complete equilibrium (Krogh and Krogh, 1910) with the alveolar air, which varies in its oxygen content from 14.5 to 17.5 per cent. Assuming a mean value of 16 per cent, the oxygen dissolved at 38°C. is approximately $100 \times 0.022 \times 0.16 \times \frac{760 - 49}{760} = 0.33$ volume per cent. In venous blood, however, and in partially saturated arterial blood, the amount of physically dissolved oxygen varies with the varying oxygen tension, which is approximately indicated by the percentage of complete saturation of the blood with oxygen. An average value for the percentage of saturation of venous blood is 65 per cent, which corresponds to an oxygen tension of 35 mm. when the CO_2 tension is normal (40 mm.). There is, therefore, $100 \times 0.022 \times \frac{35}{760} = 0.10$ volume per cent of oxygen physically dissolved in such a sample.

Therefore when the total O_2 is determined by extraction and absorption with pyrogallol, the O_2 combined with hemoglobin

may be approximately estimated by subtracting from the total O_2 0.3 volume per cent for arterial blood and 0.1 for venous.

When N_2 and O_2 are measured together, the corrections for dissolved $N_2 + O_2$ which are subtracted in order to estimate the combined O_2 are $1.36 + 0.3 = 1.66$ volumes per cent for arterial, and $1.36 + 0.10 = 1.46$ for venous blood. Discarding the last decimals, the corrections are 1.7 volumes per cent for arterial blood, 1.5 for venous.

The above corrections are estimated from the average oxygen tensions of arterial and venous blood. The corrections due to dissolved oxygen are so minute that fluctuations from the average can exceed the experimental error only in most refined work. If, however, a greater degree of accuracy is desired the values in Table IV may be used. They are calculated from the curve of Barcroft (1914).

C. Oxygen Combined with Hemoglobin in Blood Artificially Saturated with Air (Oxygen Capacity of Blood).—In determining the oxygen capacity (usually as a measure of the hemoglobin content) blood is saturated with air at room temperature, as described in the original paper (Van Slyke, 1918). From the total $N_2 + O_2$ obtained on extraction of blood thus saturated, it is necessary to deduct the N_2 and the physically dissolved O_2. According to Bohr (1905) the solubility coefficient of oxygen in blood is 0.031 at 15°, 0.022 at 38°. For the usual room temperature range of 15–25°, the coefficient varies from 0.031 to 0.027, with a mean of 0.029 at 20°. The volume per cent of oxygen physically dissolved by blood equilibrated with atmospheric air at 20°, 760 mm., may therefore be estimated as $100 \times 0.029 \times 0.209 \times \frac{760 - 17}{760} = 0.59$. At 25° the figure would be 0.55, at 15° it would be 0.63. The figure thus calculated from the solubility coefficient may, as in the case of nitrogen, not be exact, but it does not appear probable that the error, if there is one, is large enough to be significant. If we add the figure for oxygen dissolved at 20° to the empirically determined nitrogen content of blood saturated with air at room temperature we have $0.59 + 1.52 = 2.11$ volumes per cent of physically dissolved $O_2 + N_2$ to subtract from the total $O_2 + N_2$ content in order to obtain the oxygen combined with hemoglobin.

When the total O_2 of the saturated blood is determined directly, by absorption with pyrogallol, correction is to be made only for the 0.59 volume per cent of physically dissolved oxygen.

In Table I of the original article (Van Slyke, 1918) the volume of "dissolved air" to be subtracted from blood shaken at 20° was calculated from the solubility coefficient of air to be 0.034 cc. This corresponds to 0.031 cc. measured at 0°, or 1.55 volumes per cent of the 2 cc. of blood analyzed, a figure 0.55 volume per cent below that based on the nitrogen content which we find by actual analysis. Consequently oxygen capacities calculated as outlined in the original paper have been approximately 0.55 volume per cent too high. This corresponds to an error of plus 0.41 gm. of hemoglobin per 100 cc. of blood, or plus 3.0 per cent of the average normal hemoglobin content.

TABLE IV.

Oxygen Estimated Held in Physical Solution by Blood at 38° at Different Oxygen Tensions and Saturations.

Saturation of blood with oxygen.	Oxygen tension corresponding to saturation in Column 1.	Oxygen physically dissolved at 38°C.	Total correction $N_2 + O_2$ (dissolved).
per cent	*mm. Hg*	*vol. per cent*	*vol. per cent*
0	0	0.00	1.36
10	11	0.03	1.39
20	16	0.04	1.40
30	19	0.05	1.41
40	22	0.07	1.43
50	27	0.08	1.44
60	31	0.09	1.45
70	37	0.11	1.47
80	46	0.13	1.49
90	65	0.18	1.54
95	90*	0.24	1.60
100	120	0.32	1.68
100	140†	0.37	1.73
100	150‡	0.40	1.76

* Approximate arterial tension.

† Tension of blood equilibrated *in vitro* at 38° with air containing CO_2 at 40 mm.

‡ Tension of blood equilibrated *in vitro* at 38° with air containing no added gas.

D. Calculation of the Oxygen Unsaturation of Blood.—The oxygen unsaturation of blood was first defined by Lundsgaard (1918)

as the difference between the oxygen content of blood and the oxygen capacity. In determining the oxygen unsaturation Lundsgaard and others, including the authors, have estimated the oxygen capacity as the oxygen content of blood saturated with air at room temperature. At average atmospheric conditions (20° and 143 mm. oxygen tension) blood containing sufficient hemoglobin to bind 20.00 volumes per cent of oxygen would contain 20.58 volumes per cent of total oxygen, the extra 0.58 being the amount held in physical solution. However, at 38° the same blood would likewise bind 20.00 volumes per cent of oxygen with its hemoglobin, but would dissolve physically only 0.32 volumes per cent, making a total of 20.32 volumes per cent. The oxygen unsaturation of such a blood would be calculated according to the above mentioned method as 20.58 − 20.32 = 0.26 volume per cent, instead of zero, which it obviously is. The error of calculation is barely within the limit of experimental error of the determinations, and is altogether too slight to affect significantly any results thus far published, but it seems nevertheless worth while to correct it in the future by basing the calculations on figures representing oxygen combined with hemoglobin rather than on total oxygen. The example given at the end of the paper indicates the preferred mode of calculation.

Table XIII at the end of this paper will be found of convenience in making the above calculations.

3. Magnitudes of Error in Different Steps of the Oxygen Determination.

A. Measurement of Blood Sample.—The accuracy with which samples of blood can be delivered from an Ostwald pipette of the type described by Van Slyke and Cullen (1914, p. 215) is indicated by the following successive weighings. The blood was well stirred before each sample was drawn. Nine successive weighings from a 2 cc. pipette gave 2.1072, 2.1090, 2.1100, 2.1076, 2.1093, 2.1071, 2.1072, 2.1051, and 2.1069 gm. of blood, the maximum variation being from 2.1101 to 2.105, or 2.1075 ± 0.0025 gm., the maximum deviation, 0.0025 gm. being 0.11 per cent, the average 0.05 per cent of the amount measured.

B. Reading of Gas Volume in Fine Bore Apparatus with 0.1 to 0.2 Cc. of Water above Mercury. Levelling Bulb Adjusted with

Eye. No Side Arm or Levelling Scale.—A series of measurements on the same portion of gas under the above conditions gave, when reduced to 760 mm., 0°, eight successive readings between 0.729 and 0.732 cc., the maximum deviation being, therefore, 0.0015 cc. from the mean, or 0.2 per cent of the gas volume measured.

C. Reading of Gas Volume in Fine Bore Apparatus with 8.5 Cc. of Water above Mercury. Side Arm on Levelling Bulb. New Levelling Scale (Stadie, 1921) Used in Adjusting Levelling Bulb.—Nine successive readings on a given gas sample varied between 0.884 and 0.882 cc., the maximum deviation being, therefore, 0.001 cc. from the mean, or 0.1 per cent of the gas volume measured. Without the Stadie levelling scale, a maximum variation of ±0.004 cc., or four times as great, was encountered.

D. Reabsorption of Air by Reaction Mixture During Delivery of Blood.—When, after being freed of air, the reaction mixture is forced up into the cup it is exposed to the atmosphere for a period of 1 to 4 minutes while the blood is being stirred and pipetted into the apparatus. Repeated tests have shown that no measurable amounts of air are absorbed by the reaction mixture during even longer periods of quiet exposure to the atmosphere.

E. Constancy of Results Obtainable.—With the use of the levelling scale and the technique described above the volumes of $O_2 + N_2$, reduced to 0°, 760 mm., obtained from 2 cc. samples of blood were the following:

Blood 1, 0.555, 0.555, 0.555, and 0.556 cc.

Blood 2, 0.436, 0.436, 0.442, 0.433, and 0.436 cc.

The first series represents somewhat exceptional constancy, but the second, with a maximum variation of 0.437 ± 0.005 cc., or ± 0.25 volume per cent of oxygen, indicates rather greater variations than are usually to be expected.

4. Relative Yields of Oxygen from Blood Laked in Alkali, Water, and Acid.

The fact that a sufficient grade of alkalinity diminishes the yield of oxygen, and that acid may to a less extent cause a similar diminution is indicated by the data in Table V. The analyses were performed as described on page 13 of this paper, except that instead of 6 cc. of water added to the 2 cc. sample of blood,

an equal volume of a solution containing the indicated amounts of acid or alkali was added. The extracted gas was tested with NaOH solution to absorb CO_2 after use of the alkaline as well as the acid and neutral solutions. An amount of CO_2 equivalent to 0.5 volume per cent of the blood was obtained from the blood + ammonia mixture, and to 0.65 from the carbonate.

TABLE V.

Oxygen Content of Blood Determined after Laking in Alkali, Water, and Acid, Respectively.

Alkali or acid added.	Amount of acid or alkali per cc. of blood.	$O_2 + N_2$.		CO_2
	millimols	*vol. per cent*	*per cent of maximum*	*vol. per cent*
Na_2CO_3	0.28	25.15	93.6	0.55
NH_3	0.15	26.35	98.1	0.65
NaOH	0.04	21.30	79.3	
NaOH	0.02	26.40	98.3	
NaOH	0.01	26.85	100.0	
None	0.00	26.80	99.8	
Lactic acid	0.01	26.85	100.0	
" "	0.06	26.65	99.3	
" "	0.10	26.35	98.1	

The results with this blood seem to indicate that the maximum yield of oxygen is obtained when water with negligible amounts of acid or alkali is used. That other bloods do not consistently yield higher oxygen contents when *no* acid or alkali is added is indicated by Table VI, however.

That the variations in results with the different reagents were not due to analytical errors appears to have been excluded by the constancy with which duplicates with each solution agreed. Blood 3 was controlled with particular care. One sample each was analyzed in ammonia, water, lactic acid, and sodium carbonate solution in the order mentioned, then the entire series was repeated three times in the same order. The possibility of a change in the oxygen content of the blood, such as might have been conceived had all the ammonia solutions been done together, then all the water solutions, etc., was excluded. The results obtained were, nevertheless, in cc. of gas reduced to 0°, 760 mm.,

from 2 cc. of blood, the following: ammonia, 0.431, 0.431, 0.435, 0.433, average 0.433; water, 0.430, 0.430. 0.430, 0.431, average 0.430; lactic acid, 0.415, 0.408, 0.409, 0.417, average 0.410; carbonate, 0.409, 0.410, 0.410, average 0.410.

The variations might be explainable on the basis of the previously mentioned oxygen reabsorption if the alkaline solutions were regularly lower, but they are not. All that one can conclude at present is that the maximum, or nearly maximum amount of oxygen is yielded by blood treated with ferricyanide in water solution; and that markedly increasing the alkalinity greatly reduces the yield. The amounts of ammonia hitherto used, 0.15

TABLE VI.

Oxygen Contents of a Series of Bloods Determined after Laking in Alkali, Water, and Acid, Respectively.

Blood No.	Species.	$O_2 + N_2$ content.			
		Ammonia 0.225 millimol per cc. of blood.	Water.	Lactic acid 0.1 millimol per cc. of blood.	Na_2CO_3 0.28 millimol per cc. of blood.
		vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	Horse ++	17.58	17.70	17.65	
2	Man ++	20.73	20.91	20.95	
3	Dog ++++	21.5	21.5	20.5	20.5
4	Dog ++	21.5	20.9	20.5	
5	Man ++	23.2	23.7	23.6	

++ Two agreeing determinations with each reagent.
++++ Four " " " " "

to 0.20 millimols per cc. of blood, may in some bloods cause variations of a fraction of a volume per cent of oxygen from the results with water; and similar variations may be caused by the amount of lactic acid (0.1 millimol per cc.) required to free all the CO_2.

5. Comparison of Results of Oxygen Capacity Determinations by Present Method and Haldane Method.

Comparative determinations were carried out in a series of five bloods by our method as described in this paper, with water to lake the blood, and by Haldane's recent modification of his method. The results given in Table VII are each the average of

two or more determinations. The results by Haldane's method average 1 volume per cent lower than those by ours. It appears probable that the relatively low results by Haldane's method are due to the difference in the laking solutions used, rather than to the difference in apparatus employed to measure the oxygen evolved, since when instead of water we used 1 per cent Na_2CO_3 solution (as in the Haldane method) to lake the blood in our own apparatus we encountered similarly low results.

TABLE VII.

Oxygen Capacities of a Series of Bloods Determined by the Authors' and by Haldane's Method.

No.	Authors' method.	Haldane method.	
	vol. per cent O_2	*vol. per cent* O_2	*per cent of capacity by authors' method*
1	21.78	19.80	90.9
2	21.84	20.84	95.4
3	19.37	19.62	101.2
4	19.62	18.75	95.6
5	15.29	14.30	93.6
Average.			95.3

Determination of Carbon Dioxide in Whole Blood and Plasma.

The following changes have been made in the original methods.

1. Transfer of Blood into Apparatus.—The 1 per cent ammonia solution used for washing out the cup of the apparatus[3] before each determination has been dispensed with. Unless kept with especial precautions it gradually absorbs carbon dioxide from the air, and consequently must be continually tested. Otherwise a few drops left in the apparatus may carry enough carbon dioxide to make a measurable plus error in the results.

At present we merely rinse the cup out with water before each determination, and then run into it 1 cc. of distilled water. The blood or plasma is run under this layer of water. Even if the latter has an acid reaction it affords an efficient mechanical hindrance to loss of carbon dioxide from the blood in the short time that the latter is in the cup. A drop of octyl alcohol is

[3] Van Slyke (1917), p. 354.

added, and the blood or plasma, followed by the layer of water, is run into the chamber of the apparatus. The layer of water, thus following the blood, serves to wash down the walls of the cup. In the case of whole blood, some corpuscles settle on the bottom of the cup. These are suspended in the water by stirring them up with the last 0.5 cc. of water after the first 0.5 cc. has passed into the chamber.

After the blood or plasma, water, and octyl alcohol in successive layers have run into the apparatus, leaving only the drop of alcohol in the capillary above the cock, the 0.5 cc. of acid is measured into the cup and run through into the chamber.

2. Lactic Acid Instead of Sulfuric for Decomposing the Blood Bicarbonate.—With plasma, sulfuric acid is entirely convenient, but when sulfuric acid is added to whole blood it causes a heavy protein precipitate, which adds some difficulty to accurate reading and subsequent cleaning of the apparatus. L. L. Van Slyke and Baker (1919) encountered a similar difficulty with casein precipitates in using the apparatus to determine carbon dioxide in milk, and obviated it by substituting lactic acid for sulfuric, the lactic holding the protein in solution instead of precipitating it. We have found that a similar advantage is gained by substituting N lactic acid for N sulfuric in the analysis of whole blood. The hemoglobin forms a clear burgundy solution with a smooth meniscus to read, and after the analysis is completed the apparatus may be cleaned by simply rinsing with water. Lactic acid is made up with sufficient accuracy for the purpose by diluting 1 volume of concentrated acid (specific gravity 1.20) to 10 volumes with water.

3. Absorption of CO_2 in Analysis of Whole Blood.—For absorption of the CO_2 in the mixture of gases obtained after extraction of whole blood we find 0.5 N NaOH preferable to the 10 per cent KOH recommended previously.[4] The more dilute alkali is of such light specific gravity that it floats on top of the blood, and makes a clear meniscus for reading the final volume. It should be admitted into the chamber with *slight* negative pressure, the mercury in the levelling bulb being held a few cm. below that in the chamber. If it is held much lower a slight amount of air may be extracted from the NaOH solution.

[4] Van Slyke (1917), p. 356.

4. Measurement of CO_2 without Removal of Blood Solution from the Measuring Chamber.—When the manipulations are properly performed, it is possible to leave out one step in the analysis; *viz.*, running the solution out of the main chamber into the lower bulb before releasing the vacuum. The vacuum may be released and the gas volume read with all the solution in the chamber, and yet without increased absorption of CO_2, if the following procedure is followed. After extraction of the gas the lower cock is opened, admitting the mercury into the extraction chamber rapidly until the meniscus of the water solution reaches the contracted upper portion of the chamber. At this moment the lower cock is partially closed, and the remainder of the mercury is admitted at a rate sufficiently retarded to *prevent oscillation of the water column* in the calibrated portion of the apparatus, when pressure equilibrium is reached. The pressure is then adjusted by placing the mercury surface in the levelling bulb above the mercury meniscus in the chamber by a height equal to 1/13 that of the water column, in order to balance the latter. After some practice controlled by a centimeter rule, one can estimate this level with the eye to within 2 mm. of mercury, which is sufficiently accurate for many purposes. A more accurate practice, however, is to use the levelling scale described by Stadie (1921) and avoid the possibility of any error whatever in regulating the pressure. When the pressure has been adjusted the lower cock of the apparatus is closed. The gas volume may then be read at leisure.

5. Cleaning Apparatus after Determination of CO_2 in Whole Blood.—When, as in most plasma analyses, the CO_2 is not reabsorbed no washing of the apparatus is necessary before using it for another determination, since the acid solution which wets the walls of the chamber contains a negligible amount of CO_2. When the CO_2 is reabsorbed by alkali solution, however, as in analysis of whole blood, the solution retains all the absorbed CO_2 and must be completely washed out before another analysis is performed. The washing is conveniently carried out as follows: The upper cock of the apparatus is closed, and the levelling bulb dropped to its lowest level. While the mercury in the apparatus is falling, the 6 cc. cup at the top is filled with water. By this time the apparatus is evacuated and nearly all the water from the cup is let in, washing the entire chamber. The water is ejected,

and the rinsing repeated once more, 1 cc. of water being left in the cup to start the next analysis. Washing the apparatus out in this manner consumes less than a minute.

6. Correction for CO_2 Reabsorbed After Release of Vacuum.

While the mercury is rising in the apparatus during release of the vacuum after extraction of CO_2, the layer of water over the mercury surface has an opportunity to reabsorb some of the CO_2 gas. In the original method (Van Slyke, 1917) such reabsorption was guarded against by withdrawing the water solution as completely as possible into the bulb below the lower cock before the vacuum was released. By this expedient the volume of the water layer left on the mercury was reduced to 0.01 to 0.02 cc. Control analyses with standard solutions of Na_2CO_3 gave theoretical results, so that it seemed that the error due to reabsorption had been made negligible.

We have found, however, that although small, it is not negligible. The film of water as it rises in the apparatus reabsorbs 1.5 to 2 per cent of the total amount of CO_2 present. The fact that in the former control analyses 100 per cent instead of 98 to 98.5 per cent of the theoretical amount of CO_2 was obtained from standard Na_2CO_3 solutions was presumably due to the presence of a slight excess of CO_2 absorbed from the laboratory air by the strongly alkaline standard stock solution during the interval between its preparation and the analyses. Such absorption may occur more rapidly than at the time was realized.

Concerning the reabsorption of CO_2 in the apparatus after release of the vacuum, we have ascertained the following.

a. The amount of CO_2 reabsorbed is a constant proportion of the amount present.

b. The reabsorption occurs almost entirely during the last part of the compression, while the gas volume is undergoing reduction from 5 cc. to its final volume.

c. It is independent of the volume of water solution on the surface of the mercury. The absorption apparently is confined so nearly to the surface during the short period involved that depth of layer has relatively slight influence. The volume of water solution may be varied from 0.10 to 2.5 cc. without varying the volume of CO_2 reabsorbed.

d. Variations within wide limits (*e.g.* 5 and 40 seconds) in the period during which the mercury is allowed to rise have no appreciable influence on the volume of CO_2 reabsorbed.

e. The one factor that may markedly increase the proportion of CO_2 reabsorbed, is the manner in which the meniscus of water over mercury is brought to rest at atmospheric pressure in the measuring tube. If, as the meniscus approaches the narrower upper part of the apparatus, its rise is retarded, and the surface is allowed to come gently to the point of equilibrium with no oscillation, a minimum and constant proportion of CO_2 (1.7 per cent of the total) is reabsorbed. On the other hand, if the final rise is permitted to be violent, and the mercury with the water above it in consequence oscillates several times to a distance of several mm. above and below the point corresponding to atmospheric pressure before it comes to rest, 0.01 to 0.02 cc. of CO_2 may be reabsorbed in the process.

Certain of the above points are demonstrated by the following experiments.

Analyses of Standard Na_2CO_3 Solutions.—Na_2CO_3 was prepared from Merck's "Reagent" $NaHCO_3$ by heating for several hours in an oven at 250°C. The Na_2CO_3 thus prepared was kept in bottles with glass stoppers that had been rendered tight by regrinding and greasing.

A 0.03 molecular solution of Na_2CO_3 (3.180 gm. Na_2CO_3 per liter) was prepared by weight, and checked by titrating against 0.1 N HCl. 20 cc. neutralized to methyl orange 11.97 and 12.04 cc. of 0.1 N HCl, after subtraction of 0.10 cc. of 0.1 N HCl required to give the chosen end-point in a control water solution. Calculated 12.00 cc.

The CO_2 content was determined with an apparatus of the usual size (50 cc. chamber, 1 cc. measuring scale), but designed like the micro-apparatus described by Van Slyke (1917, p. 363), so that there was no chance for reabsorption of CO_2 during release of the vacuum. Found, 67.4, 67.0, and 67.2 volumes per cent CO_2. Calculated 67.2. Found = 100.0 per cent of calculated.

The CO_2 content of this solution was redetermined on 1 cc. samples with the ordinary macro-apparatus (Van Slyke, 1917, p. 349) with a single extraction. Found, 65.5, 66.2, 65.9, 66.2,

66.4, average 66.0 volumes per cent. Found = 98.2 per cent of calculated.

A 0.015 M solution was prepared by dilution of the above 0.03 M solution with CO_2-free water, and the CO_2 was determined by a single extraction in the same apparatus. Found, 33.1, 33.1, 33.1, and 33.1 volumes per cent of CO_2. Calculated 33.6. Found = 98.5 per cent of calculated.

Direct Determination of Reabsorbed CO_2.—The determinations were carried out in the fine bore macro-type apparatus (Fig. 1) and were performed in the usual manner up to the point at which the vacuum is released. The mercury was allowed to rise to the desired point. At this point the outlet capillary, *a*, was connected with a suction pump. Then the supernatant atmosphere was freed of CO_2 by applying suction, letting in air, and applying suction again, by revolving the upper cock. This operation was performed in a few seconds, during which the solution in the chamber was kept quiet, in order to avoid reextracting the CO_2 that it held in solution. The air was then driven out through the cup at the top, the solution being driven after it into the cup, where it remained for a few seconds while the mercury seal of outlet, *a*, was reestablished. The solution was then readmitted into the chamber, and its CO_2 was extracted and measured. The measurements of the small volumes of gas were made after reducing the pressure by 500 mm. of mercury, as described in this paper, and the purity of the CO_2 was controlled by absorption with NaOH. The results are given in Table VIII.

From the preceding analyses of standard carbonate solutions we have shown that one extraction by the usual technique yields 98.3 to 98.5 per cent of the calculated CO_2 volume, while from the analysis, in Table VIII, of the untrapped solution remaining in the chamber we have recovered 1.5 to 2.2, on the average 1.7 per cent of CO_2 reabsorbed during the rise of the mercury to the point of establishment of atmospheric pressure (Determinations 9 to 15). It is therefore demonstrated in two ways that reabsorption diminishes by a quite constant proportion, *viz.* 1.7 per cent, the amount of CO_2 obtained by the single extraction method used as originally described by Van Slyke. The constancy of the correction justifies its introduction into the calculations by

multiplying the theoretically established factors by $\frac{1}{0.983} = 1.017$.
This we have done in Table XIII.

Alternatives to using this correction factor are: (1) To re-extract the untrapped water solution after each analysis, as described in the experiments reported in Table VIII, and (2) to use

TABLE VIII.

Determination of Reabsorbed CO_2.

No.	*a* CO_2 in Na_2CO_3 solution analyzed.	*b* Volume of free space left in chamber over Hg when CO_2 was replaced by air.	*c* Volume of untrapped solution left in chamber.	*d* CO_2 obtained from 2nd extraction of untrapped solution.	*e* CO_2 estimated left in untrapped solution after 1st extraction.*	*f* CO_2 reabsorbed after 1st extraction: $c - d$	*f* CO_2 reabsorbed after 1st extraction: $100 \frac{c-d}{a}$
	cc.	cc.	cc.	cc.	cc.	cc.	*per cent of total*
1	0.672	50	0.17	0.002	0.002	0.000	0.0
2	0.672	50	0.25	0.004	0.003	0.001	0.1
3	0.672	25	0.29	0.004	0.004	0.000	0.0
4	0.672	5	0.20	0.005	0.003	0.002	0.3
5	0.672	5	2.50	0.033	0.030	0.003	0.4
6	0.672	2	0.20	0.007	0.002	0.005	0.7
7	0.672	1	0.16	0.012	0.002	0.010	1.5
8	0.672	1	0.10	0.012	0.001	0.011	1.6
9	0.672	0.78	0.18	0.014	0.003	0.011	1.6
10	0.672	0.78	0.20	0.014	0.003	0.011	1.6
11	0.672	0.74	0.18	0.013	0.003	0.010	1.5
12	0.336	0.40	0.10	0.007	0.001	0.006	1.8
13	0.336	0.40	0.20	0.009	0.0016	0.0084	2.2
14	0.336	0.40	2.50	0.024	0.018	0.006	1.8
15	0.336	0.40	2.50	0.023	0.018	0.005	1.5

* Calculated as $a\ c \frac{\alpha'_{CO_2}}{A + (\alpha'_{CO_2} - 1) S}$

the micro-apparatus (Van Slyke, 1917) in the original or an enlarged form. In such an apparatus the water solution may be entirely separated from the gas phase before the vacuum is released, and reabsorption of CO_2 therefore made impossible.

We have found, however, that with care to prevent oscillation of the mercury-water layer after atmospheric pressure is reached, such constant results are obtained with the simpler, usual apparatus and technique (see Table I), that we prefer to continue its use, merely introducing the factor 1.017 to correct for the reabsorp-

tion. This correction involves a change of about 1 volume per cent in the results of an ordinary blood or plasma CO_2 determination.

7. Calculation of CO_2 Results.—In deriving the formula by which results were calculated in the original paper (Van Slyke, 1917, p. 358) the factor $\frac{S}{50}\alpha'_{CO_2}$ was used to calculate the fraction of the gas remaining dissolved in the water when equilibrium was reached in extraction. (S = cc. of water solution, 50 = cc. volume of extraction chamber, α'_{CO_2} = cc. of CO_2, measured at the prevailing temperature, dissolved by 1 cc. of water in equilibrium with CO_2 gas at 760 mm. tension.) This factor represents an approximation that is entirely exact only when $\alpha'_{CO_2} = 1$, which occurs at 18°. The deviation between the above approximate solubility correction factor and the exact factor increases, (1) as α'_{CO_2} becomes greater or less than 1, and (2) as the ratio $\frac{S}{50}$ increases. For the conditions under which the approximate factor was used, however, (temperature range $15-30°, \frac{S}{50} = 0.05$) the error introduced does not exceed 1 part per 1,000. Consequently the numerical factors arrived at by the original approximate equation do not require correction for the sake of accuracy.

It seems, however, desirable to present the exact general equation which expresses the relationships between gas and liquid phases under conditions such as those prevailing in the apparatus.

V_t = volume of CO_2 obtained by one extraction and measured at atmospheric conditions of $t°$ temperature and B mm. barometric pressure.

$V_{0°, 760}$ = total volume of CO_2, reduced to 0°, 760 mm., in the solution analyzed.

S = volume of water solution in apparatus.

A = volume of chamber occupied by gas and solution during extraction (50 cc. in our apparatus).

$A - S$ = volume of gas phase during extraction.

T = absolute temperature, $= t + 273$.

α_{CO_2} = solubility coefficient of CO_2 in water, the cc. of CO_2 measured at 0°, 760 mm., dissolved by 1 cc. of water in equilibrium with CO_2 under 760 mm. tension.

$\alpha'_{CO_2} = \alpha_{CO_2} \times \frac{T}{273}$ = distribution coefficient of CO_2 between gas and water = cc. of CO_2 measured at $t°$, B mm. dissolved by 1 cc. of water in equilibrium with pure CO_2 at $t°$, B mm. (α'_{CO_2} was referred to as α_{CO_2} in the original paper.)

w = vapor tension of water.

p = partial pressure of CO_2 in apparatus when equilibrium is reached in the extraction.

x = volume of CO_2 gas, measured at 0°, 760 mm., held in solution when equilibrium is reached.

The total CO_2 content of the solution analyzed is obtained by reducing the volume of CO_2 extracted to standard conditions by multiplication with the usual factor $\frac{(B - w)\,273}{760\,T}$, and adding to the volume thus corrected the volume x of CO_2 remaining in solution. Thus:

$$V_{0°,\,760} = V_t \frac{B - w}{760} \times \frac{273}{T} + x \tag{1}$$

Since the volume of gas dissolved is proportional to its partial pressure, solubility, and the volume of the solvent, we have

$$x = \frac{p}{760} S\alpha_{CO_2} \tag{2}$$

Since pressure varies inversely as gas volume

$$\frac{p}{B - w} = \frac{V}{A - S}, \text{ or} \tag{3}$$

$$p = (B - w)\frac{V}{A - S} \tag{4}$$

Substituting this value for p in (*2*), and the value thereby found for x in (*1*), we have

$$V_{0°,\,760} = V_t \frac{B - w}{760} \times \frac{273}{T} + V\frac{B - w}{760} \times \frac{S\alpha_{CO_2}}{A - S} \text{ or} \tag{5}$$

$$V_{0°,\,760} = V_t \frac{B - w}{760}\left(\frac{273}{T} + \frac{S\alpha_{CO_2}}{A - S}\right) \tag{6}$$

Since $\alpha_{CO_2} = \alpha'_{CO_2} \times \frac{273}{T}$, equation (*6*) may be expressed as

$$(7) \qquad V_{0°, 760} = V_t \frac{B - w}{760} \times \frac{273}{T} \left(1 + \frac{S}{A - S} \alpha'_{CO_2}\right), \text{ or}$$

$$(8) \qquad V_{0°, 760} = V_t \underbrace{\frac{B - w}{760\ (1 + 0.00367\ t)}}_{\substack{\text{Factor correcting for}\\ \text{atmospheric pressure}\\ \text{and temperature.}}} \underbrace{\left(1 + \frac{S}{A - S} \alpha'_{CO_2}\right)}_{\substack{\text{Factor correcting}\\ \text{for unextracted}\\ CO_2.}}$$

In the approximate equation used in the original paper the factor correcting for unextracted CO_2 was $\frac{1}{1 - \frac{S}{A} \alpha_{CO_2}}$. When $\alpha'_{CO_2} = 1$ (at 18°) this becomes identical with $1 + \frac{S}{A - S} \alpha'_{CO_2}$, both factors then reducing to $\frac{A}{A - S}$.

The values of the combined factor $\frac{B - w}{760\ (1 + 0.00367\ t)} \left(1 + \frac{S}{A - S} \alpha'_{CO_2}\right)$, multiplied by 1.017 to correct for reabsorption of CO_2 during release of the vacuum, are given in Table XIII at the end of this paper for the calculation of results. Expressing $\frac{B - w}{760\ (1 + 0.00367\ t)}$ as f, we have given its values for $B = 760$, to be multiplied by $\frac{B}{760}$ for other barometric pressures. Because the vapor tension, w, is not quite the same fraction of $B - w$ when B is other than 760, this usage is not absolutely exact. At a barometric pressure $B = 740$ it introduces an error of plus 0.1 per cent, at 700 mm., an error of plus 0.4 per cent. For barometric pressures outside the range 740 to 780 mm., therefore, one must use the customary tables for values of $\frac{B - w}{760\ (1 + 0.00367\ t)}$ in order to avoid errors exceeding 1 part per 1,000. For work at ordinary altitudes, however, the factors as given in Table XIII are sufficiently exact.

Determination of Carbon Monoxide.

In the method of Van Slyke and Salvesen (1919) it appears slightly more accurate to use 1.36 volumes per cent as the correction for nitrogen gas rather than the 1.2 volumes per cent correction found by the above authors. Still more accurate results may be obtainable by absorption of the CO with ammoniacal, cuprous chloride solution as recently described by O'Brien and Parker (1921).

In the determination of carbon monoxide it is advisable to use the finer bore apparatus and to magnify the gas volumes by reducing the pressure as described at the beginning of this paper, since the amounts of gas measured are likely to be much smaller than those measured when carbon dioxide or oxygen is determined.

The blood should be trapped in the lower bulb of the apparatus before releasing the vacuum, as it is undesirable to mix blood with pyrogallol and cuprous chloride.

Determination of Methemoglobin.

In the determination of methemoglobin (Stadie, 1920) the total blood pigment is determined colorimetrically, and the methemoglobin is estimated by subtracting from the total pigment the oxyhemoglobin. The latter is determined by the oxygen capacity method. The oxygen capacity portion of the methemoglobin determination therefore requires revision in its method of calculation, as outlined above. Instead of utilizing Table I of Stadie's paper the oxygen capacity of the blood is determined and calculated as described above, to make complete allowance for the nitrogen gas content of the blood. The volume per cent of oxygen is then multiplied by 0.746, the number of gm. of hemoglobin that combines with 1 cc. of oxygen. The result is the number of gm. of hemoglobin per 100 cc. of blood.

The change in the mode of calculation affords sufficient increase in accuracy to be justified, but the error involved in the former method is too slight to invalidate any results that have been obtained by it. The absolute error is 0.5 volume per cent in oxygen capacity (equivalent to 0.37 gm. of hemoglobin per 100 cc. of blood) or about 2.5 per cent of the amount normally present. In the methemoglobin calculation this error is partly neutralized by the fact that the same percentage error is intro-

duced into the colorimetric estimation of total pigment by making the standard methemoglobin solution from blood in which the hemoglobin had been estimated by the same oxygen capacity method. For example, in Table IX the model calculation given on page 240 of Stadie's paper (1920) is reproduced together with the calculation as corrected in this paper. It is seen that the change in methemoglobin calculated is but 0.1 gm. per 100 cc. of blood.

TABLE IX.

Methemoglobin Calculation.

	Hemoglobin per 100 cc. of blood.	
	Calculated as in original paper (Stadie, 1920).	Calculated as described in this paper.
	gm.	*gm.*
Hemoglobin strength of standard blood calculated from oxygen capacity	15.0	14.6
Total blood pigment colorimetrically determined = $\frac{10}{12}$ of standard	12.5	12.2
Hemoglobin determined by oxygen capacity	10.0	9.6
Methemoglobin by difference	2.5	2.6

Determination of All the Gases in One Blood Sample.

Both carbon dioxide and oxygen, as well as the residual nitrogen, may be determined in one sample of blood with but little more expenditure of time and effort than is required to determine any one of the gases alone. For liberation of the gases both acid and ferricyanide are added to the blood in the apparatus, and all the gases are extracted and measured together. The CO_2 is then absorbed by dilute alkali, leaving O_2 and N_2. The O_2 may then be determined by absorption with pyrogallol, or estimated by subtracting the average N_2 content of blood from the sum of $O_2 + N_2$.

The essential point in determining the gases together was found to lie in the use of proper amounts of the reagents employed to set free the carbon dioxide and oxygen, particularly in the use of a minimum amount of acid. If potassium ferricyanide and a large excess of acid are added to blood, both oxygen and carbon dioxide are quantitatively freed, but the ferricyanide forms such a heavy precipitate with the blood proteins that it is inconven-

ient, although possible, to handle the resulting suspension in the apparatus. The precipitation of the proteins by ferricyanide occurs, however, only when the reaction is strongly acid. If but the minimum amount of lactic acid necessary to free the CO_2 with certainty is added, the ferricyanide-protein precipitate formed is so small in amount and so finely divided that it does not interfere with the determination. Furthermore, the acid can be trapped in the lower bulb of the apparatus before the blood is admitted, and can then be mixed with the blood after the apparatus has been evacuated. The blood is then in the lower part of the evacuation chamber, and such precipitate as does form does not touch the measuring tube at the top, which remains clean and clear. The precipitate, unlike the black deposit formed by action of ferricyanide on the mercury in the ammoniacal solution

TABLE X.

Amounts of Acid Required to Free CO_2 in Human Whole Blood.

Lactic acid added to 1 cc. of blood.	CO_2 found in blood.
millimols	*vol. per cent*
0.05	41.5
0.10	52.8
0.10	53.3
0.20	52.8

used in the original oxygen determination, is instantly soluble in 0.1 N alkali. Consequently any particles that adhere to the walls of the apparatus are removed as a rule by a single washing with dilute alkali solution.

The minimum amount of acid that will entirely free the CO_2 of whole blood for extraction under the conditions of our analyses was determined by preliminary experiments, some of which are recorded in Table X. The amount needed was found to be 1.0 cc. of 0.1 N lactic acid for 1 cc. of whole blood. Half as much fails to set the CO_2 completely free.

The minimum amount of ferricyanide required in the presence of acid is the same as in ammoniacal solution; *viz.*, 5 mg. of potassium ferricyanide per 1 cc. of normal blood (Table II). In avoiding protein precipitation, however, the amount of ferricyanide present appears to be of much less importance than the

acidity of the mixture; if the acidity is too high, the minimum necessary amount of ferricyanide causes an inconvenient precipitate, while if the acidity is not too high an excess of ferricyanide may be used without such inconvenience. We consequently employ 10 mg. of ferricyanide per cc. of blood, twice the necessary amount.

The analysis is performed as follows:

Apparatus Used.—In order to obtain with 1 cc. of blood results for carbon dioxide and oxygen accurate to within 1 per cent of the amounts measured, it is desirable to use the fine bore type of apparatus described at the beginning of this paper, although, with care, results sufficiently accurate for many purposes may be obtained with the ordinary apparatus.

Details of Determination.—First 2 cc. of 0.05 N lactic acid are admitted into the chamber of the apparatus. The acid is freed of air by evacuation and shaking in the usual manner. The air-free acid is then trapped in the bulb below the lower cock of the apparatus, and 1.9 cc. of water, with a drop of octyl alcohol, are similarly introduced and freed of air. The blood is now stirred and a sample drawn into an Ostwald pipette calibrated between two marks to deliver 1 cc. About 1.5 cc. of the air-free water in the gas analysis apparatus are run into the cup at the top, and the blood sample is at once run beneath it. We usually slightly open the cock below the cup while the pipette is draining, so that most of the blood flows directly into the chamber of the apparatus, the layer in the cup at no time being more than 2 to 3 mm. deep. The layer of water, even though it be somewhat acidified, prevents, because of the relatively slow rate of diffusion through it, the loss of CO_2 from the blood. All the blood, followed by the water layer above it, is now admitted into the chamber of the apparatus. When about half the water layer has been run in, the half remaining in the cup is stirred with a rod in order to get into suspension a few corpuscles that have lodged on the bottom of the cup. 0.05 cc. of solution containing 20 gm. of potassium ferricyanide per 100 cc. is added to this last portion of water, which is then admitted into the chamber. The 0.05 cc. of ferricyanide solution may be conveniently measured as 1 drop from a pipette, which has been found by trial to deliver thus 0.05 to 0.06 cc. of the solution.

The chamber is evacuated until the mercury has fallen to the 50 cc. mark. The 2 cc. of 0.05 N lactic acid trapped in the lower bulb of the apparatus are now admitted and mixed with the rest of the solution. At this moment a small amount of brown precipitate forms, but not enough to interfere with the subsequent manipulations. The oxygen and carbon dioxide (and carbon monoxide if present) are extracted by whirling the solution about the wall of the evacuated chamber until, when the vacuum

TABLE XI.

Comparison of Carbon Dioxide and Oxygen Contents of Normal Venous Blood Determined Separately by Former Methods, and Together by the Present Combined Method, Respectively.

Blood kept under paraffin oil. All determinations made within 3 hours after blood was drawn.

Determination.	Method.	Volume of blood for analysis.	CO_2	$O_2 + N_2$.	O_2	Potassium ferricyanide used.
		cc.	*vol. per cent*	*vol. per cent*	*vol. per cent**	*mg.*
O_2 alone.	Van Slyke, 1918.	2		21.1	19.6	40
O_2 "	" " 1918.	2		20.9	19.4	40
CO_2 "	" " 1917.	1	65.1			
CO_2 "	" " 1917.	1	65.5			
CO_2 "	" " 1917.	2	65.0			
$O_2 + CO_2$.	Present.	2	64.4	20.5	19.0	10
$O_2 + CO_2$.	"	2	65.0	20.9	19.4	10
$O_2 + CO_2$.	"	2	63.5	20.9	19.4	15
$O_2 + CO_2$.	"	2	64.4	20.9	19.4	30
$O_2 + CO_2$.	"	2	64.2	20.9	19.4	70

* The N_2 was determined and found to be 1.5 volumes per cent. It is deducted and subtracted from the $O_2 + N_2$ to estimate the O_2.

is released and the gas measured, no increase in volume is observed. Complete extraction is usually attained in 1 minute when the apparatus is shaken by hand. When the mechanical shaker devised by Stadie (1921) has been used, 2 minutes shaking after the first evacuation, followed by 30 seconds shaking for the check evacuations, has been our usual routine. As a rule no increase occurs after the first evacuation. Increase after the second indicates a leak in the apparatus, due usually to improper lubrication of the upper cock.

When extraction of the gases is complete the water solution is trapped below the lower cock, as in the original CO_2 determination, and the gases are measured at atmospheric pressure.

The gases measured are CO_2, O_2, N_2, and sometimes CO. The CO_2 is absorbed by running 0.5 N NaOH from the cup down the side of the measuring tube until no further shrinkage of the gas volume occurs; about 0.5 cc. of the hydroxide solution suffices.

TABLE XII.

Comparison of Carbon Dioxide and Oxygen Contents Determined Separately by Former Methods, and Together by the Present Combined Method.

All determinations made on 1 cc. samples in a fine bore apparatus

	Oxygen.		CO_2	
	Method.			
	Former.	Present.	Former.	Present.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
Blood aerated with alveolar air........	18.1	17.9	58.7	58.2
" diluted with saline..............	9.4	8.8	38.7	39.5
Venous blood about 60 per cent saturated with O_2......................	13.6	13.9	35.8	36.4
0.02625 M Na_2CO_3 (calculated 58.8 volumes per cent CO_2)................			58.7	59.1

As the volume, *S*, of solution in the apparatus is twice as great as in the method as originally described, the factors used for calculation of carbon dioxide are different. For the present conditions, with 5 cc. of total water solution extracted in the apparatus, the factors are given in Table XIII.

If no CO is present, the residual mixture left after absorption of CO_2 may be measured as $O_2 + N_2$, and from the $O_2 + N_2$ content reduced to 0°, 760 mm. (by Table XIII), the average N_2 content of blood, *viz.* 1.36 volumes per cent, may be subtracted in order to estimate the oxygen; or the oxygen may be measured directly by absorption, as described by Van Slyke and Salvesen. In the latter case about 0.5 cc. of pyrogallol solution is run in from the cup, and is followed by a little water to clean the tube and give a clear meniscus for reading.

TABLE XIII.

Factors for Calculation.

Temperature.	$f = \frac{B - w}{760\,(1 + 0.00367\,t)}$ factor by which gas measured moist at $t°$, B mm. is reduced to 0°, 760 mm.*	α'_{CO_2}	Air,† measured at room temperature and pressure, dissolved by 2.5 cc. H_2O.	Air,† measured at room temperature and pressure, dissolved by 5 cc. H_2O.	$1.017\,f\,(1 + \frac{S}{50 - S}\,\alpha'_{CO_2})$, factor by which the volume of CO_2 obtained after 1 extraction is multiplied in order to obtain the volume of CO_2, reduced to 0°, 760 mm., contained in the solution analyzed. S = 2.5 cc.	$1.017\,f\,(1 + \frac{S}{50 - S}\,\alpha'_{CO_2})$, ... S = 5.0 cc.
°C.			cc.	cc.		
15	$0.932 \times \frac{B}{760}$	1.075	0.052	0.105	$1.002 \times \frac{B}{760}$	$1.061 \times \frac{B}{760}$
16	0.928 "	1.043	0.051	0.101	0.995 "	1.053 "
17	0.924 "	1.015	0.050	0.100	0.989 "	1.046 "
18	0.919 "	0.989	0.049	0.098	0.983 "	1.038 "
19	0.915 "	0.966	0.048	0.096	0.978 "	1.030 "
20	0.910 "	0.942	0.047	0.095	0.972 "	1.022 "
21	0.906 "	0.919	0.046	0.093	0.966 "	1.015 "
22	0.901 "	0.896	0.045	0.091	0.960 "	1.008 "
23	0.897 "	0.873	0.045	0.090	0.954 "	1.001 "
24	0.892 "	0.850	0.044	0.088	0.948 "	0.993 "
25	0.888 "	0.828	0.043	0.086	0.942 "	0.986 "
26	0.883 "	0.808	0.042	0.084	0.936 "	0.978 "
27	0.878 "	0.789	0.041	0.083	0.931 "	0.971 "
28	0.873 "	0.772	0.040	0.081	0.924 "	0.964 "
29	0.868 "	0.755	0.040	0.080	0.918 "	0.957 "
30	0.863 "	0.738	0.039	0.078	0.912 "	0.950 "

* To calculate O_2 or hemoglobin when $O_2 + N_2$ volume is measured, multiply gas volume by f, to reduce to 0°, 760 mm., and by such factor as is necessary (100 when 1 cc. of blood is used, 50 when 2 cc. are used) to bring results to volume per cent basis. Then for

a. O_2 content, subtract..... 1.36 vol. per cent N_2

b. O_2 bound by hemoglobin in venous blood, subtract........... 1.5 " " " N_2 + dissolved O_2

c. O_2 bound by hemoglobin in arterial blood, subtract........... 1.7 " " " " " " "

d. O_2 bound by hemoglobin in blood saturated with air at 20°, subtract........... 2.1 " " " " " " "

TABLE XIII—*Concluded.*

Per cent of normal hemoglobin (Haldane scale) $= \frac{100\,d}{18.5} = 5.41\,d.$

Grams of hemoglobin per 100 cc. of blood $= 0.746\,d.$

Per cent of total hemoglobin saturated with $O_2 = \frac{100\,b}{d}$ or $\frac{100\,c}{d}$.

Volumes per cent O_2 unsaturation $= d - c$ or $d - b$.

b and c may be determined with slightly greater accuracy with the aid of Table IV. The values for f given in the second column are for barometric readings corrected for temperature (see remarks on p. 31). The values for α'_{CO_2} are obtained by multiplying by $1 + 0.00367\,t$ the values for α_{CO_2} given by Bohr and Bock (1891).

† The dissolved air is given as measured at room temperature. It is subtracted from the air + CO_2 volume, measured after one extraction of plasma or aqueous carbonate solution, in order to obtain the CO_2, which is then multiplied by $1.017\,f\left(1 + \frac{S}{50 - S}\,\alpha'_{CO_2}\right)$ in order to obtain the total volumes per cent of CO_2 in the solution analyzed. When whole blood is analyzed, the air correction cannot be used, because of the O_2 present, and the CO_2 must be determined by absorption with NaOH solution. The volume of gas absorbed is then multiplied by the above factor.

The factor 1.017, being empirical (see p. 27), may vary slightly for different apparatus.

If carbon monoxide is present, the oxygen must, of course, be absorbed by pyrogallol. The residual gas is the CO + 1.36 volumes per cent of N_2. The CO is calculated by reducing the mixture of CO and N_2 to 0°, 760 mm. (Table XIII), and subtracting 1.36 from the result in volumes per cent, or the CO may be absorbed by cuprous chloride (O'Brien and Parker, 1921).

The results of some determinations are given in Table XI and XII.

Examples of Calculations.

1. Total Oxygen Content of Venous or Arterial Blood.

a. From O_2 and N_2 Measured Together.

	Blood sample..........	2.00 cc.	
	$O_2 + N_2$ measured.....	0.405 "	at 25°, 750 mm.
$0.405 \times 0.888^* \times \frac{750}{760}$	$= O_2 + N_2$..............	0.3534 "	" 0°, 760 "
0.3534×50	$= O_2 + N_2$ per 100 cc. blood...............	17.73 "	" 0°, 760 "
N_2 subtracted	....	1.36 "	" 0°, 760 "
O_2 by difference	....	16.37 "	" 0°, 760 "

* Factor from second column of Table XIII.

b. From O_2 Determined by Absorption with Pyrogallol.

	Blood sample.........	2.00	cc.	
	$O_2 + N_2$ measured....	0.405	"	at 25°, 750 mm.
	N_2 after absorption of O_2..............	0.031	"	" 25°, 750 "
	O_2 by difference......	0.374	"	" 25°, 750 "
0.374×50	= O_2 per 100 cc. blood..	18.70	"	" 25°, 750 "
$18.70 \times 0.888^* \times \frac{750}{760}$	= O_2 " 100 " " ..	16.37	"	" 0°, 760 "

2. Oxygen Combined with Hemoglobin in Venous or Arterial Blood as Drawn.

a. Venous Blood.

Total O_2, calculated as above..............	16.37	cc. at	0°, 760 mm.
O_2 in physical solution....................	0.10†	" "	0°, 760 "
O_2 combined with hemoglobin.............	16.27	" "	0°, 760 "

b. Arterial Blood.

Total O_2, calculated as above..............	16.37	cc. at	0°, 760 mm.
O_2 in physical solution....................	0.32†	" "	0°, 760 "
O_2 combined with hemoglobin.............	16.05	" "	0°, 760 "

3. Oxygen Combined with Hemoglobin in Blood Saturated with Air at Room Temperature (Oxygen Capacity).

a. From O_2 and N_2 Measured Together.

	Blood sample.........	2.00	cc.	
	$O_2 + N_2$ measured....	0.495	"	at 20°, 767 mm.
$0.495 \times 0.910^* \times \frac{767}{760}$	= $O_2 + N_2$.............	0.455	"	" 0°, 760 "
50×0.455	= $O_2 + N_2$ per 100 cc. blood..............	22.75	"	" 0°, 760 "
Physically dissolved	$O_2 + N_2$ per 100 cc. blood..............	2.10	"	" 0°, 760 "
Combined	O_2 per 100 cc. blood..	20.65	"	" 0°, 760 "

b. From O_2 Determined by Absorption with Pyrogallol.

Blood sample.........	2.00	cc.	
$O_2 + N_2$ measured....	0.495	"	at 20°, 767 mm.
N_2 after absorption of O_2..............	0.033	"	" 20°, 767 "
O_2 by difference......	0.462	"	" 20°, 767 "

* Factor from second column of Table XIII.

† These figures are approximate and are the average for arterial and venous bloods. For the accurate values of oxygen in physical solution see Table III.

$0.462 \times 0.910^* \times \frac{767}{760} \times 50 =$ Total O_2 per 100

cc. blood...........	21.22	cc.	at	0°, 760 mm.
Physically dissolved O_2 per 100 cc. blood.	0.58	"	"	0°, 760 "
Combined O_2 per 100 cc. blood..............	20.64	"	"	0°, 760 "

4. Calculation of Hemoglobin Content from Oxygen Capacity.

Combined O_2 per 100 cc. blood, calculated as above, 20.64 cc. at 0°, 760 mm.

$20.64 \times 0.746 =$ hemoglobin per 100 cc. blood................... = 15.40 gm. hemoglobin.

$\frac{20.64}{18.5} \times 100 =$ per cent of Haldane's average normal hemoglobin....... = 112 per cent.

5. Calculation of Oxygen Unsaturation of Blood.

Oxygen combined with hemoglobin per 100 cc. of blood after saturation of blood with air at room temperature (oxygen capacity) (Calculation 3, *b*)...........................	20.64	cc.	at	0°, 760 mm.
Oxygen combined with hemoglobin of blood as drawn (Calculation 2, *a*)......................	16.27	"	"	0°, 760 "
Oxygen unsaturation.........................	4.37	"	"	0°, 760 "
Percentage unsaturation $= 100 \times \frac{4.37}{20.64}$.........	21.2 per cent.			

6. Calculation of Total Gas Content from Analysis of 1 Cc. of Blood as Described in This Paper.

Blood sample...............................	1.00	cc.	
a. $CO_2 + O_2 + N_2$ measured..................	0.845	"	at 25°, 757 mm.
b. $O_2 + N_2$ measured after CO_2 absorption	0.248	"	" 25°, 757 "
c. N_2 " " O_2 "	0.017	"	" 25°, 757 "
CO_2 by difference $(a - b)$...................	0.597	"	" 25°, 757 "
O_2 " " $(b - c)$...................	0.231	"	" 25°, 757 "

$0.597 \times 0.986‡ \times \frac{757}{760} \times 100 =$ CO_2 per 100 cc. blood.......... 58.6 " " 0°, 760 "

$0.231 \times 0.888 \times \frac{757}{760} \times 100 =$ O_2 per 100 cc. blood.......... 20.4 " " 0°, 760 "

$0.017 \times 0.888 \times \frac{757}{760} \times 100 =$ N_2 per 100 cc. blood.......... 1.5 " " 0°, 760 "

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* Factor from second column of Table XIII.

‡ Factor from last column of Table XIII.

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A MECHANICAL SHAKER AND OTHER DEVICES FOR USE WITH THE VAN SLYKE BLOOD GAS APPARATUS.

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When many determinations of the blood gases by the Van Slyke method are made from day to day the shaking of the apparatus by hand becomes laborious, and it appears worth while to describe a mechanical shaker which has been used successfully for over a year. In addition several other simple devices are described which have saved time and labor.

Mechanical Shaker.—The construction is made evident by reference to Fig. 1. The pulley is rotated by a small motor not shown in the diagram. The speed is regulated by a lamp and rheostat inserted in series. The shaking given to the apparatus by this device is rapid, efficient, and almost noiseless. There is little danger of breaking the apparatus. We have used two of these shakers for more than a year without such accident.

By means of a switch attached to an interval time clock, the current to the motor may be automatically shut off after the desired interval of shaking. The latter is usually 1 minute for a CO_2 determination in plasma, 2 minutes for either CO_2 or O_2 in whole blood.

The small rubber tube connected with the outlet of the upper stop-cock serves, after the analysis has been completed, to carry the expelled solutions to a waste bottle. A few drops of mercury are usually driven out with each portion of waste solution. The mercury thus lost from the apparatus collects in the bottom of the waste bottle, from which it is from time to time recovered, washed, strained through chamois skin, and restored to the levelling bulb.

The bottle which serves to collect the waste residue may be conveniently placed in a sink, under an aspirator suction pump,

the water from which washes the mercury. The suction may also be used conveniently to draw out waste fluids from the cup of the apparatus.

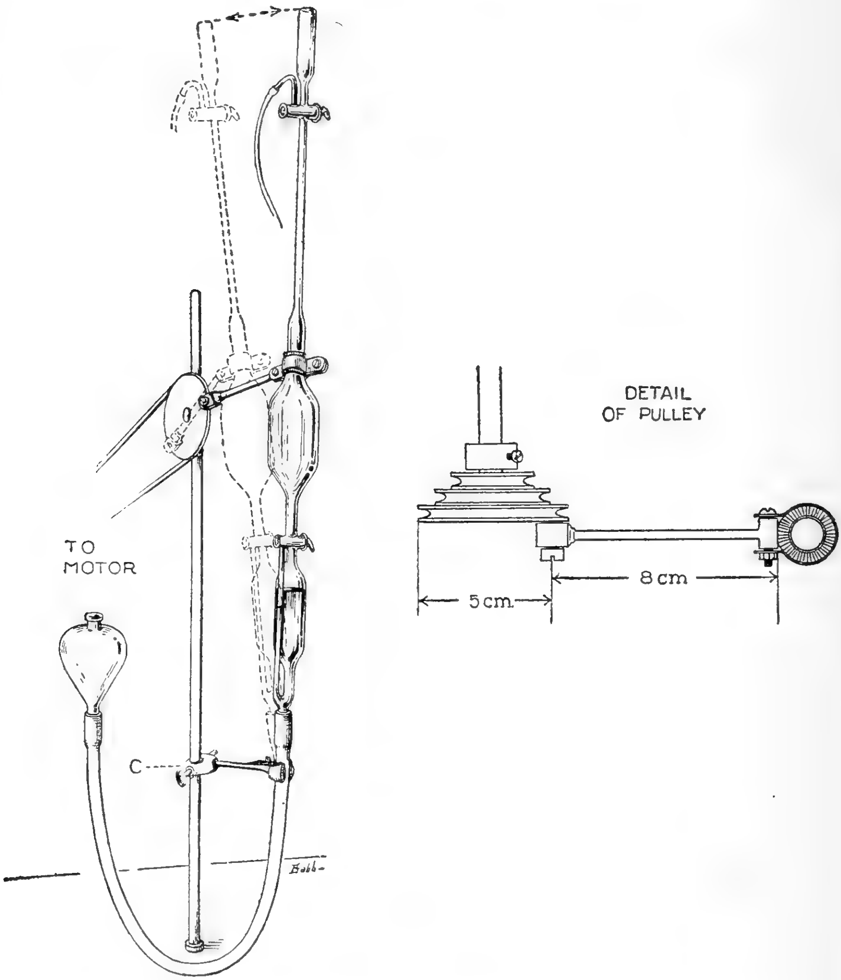

Fig. 1.

Levelling Scale.—When, as is usually our practice in oxygen determinations, the reaction mixture is left in the main chamber of the apparatus while the gas is measured, the surface of the

mercury in the levelling bulb must be raised sufficiently above that in the apparatus to balance the column of water solution in the latter. For accurate pressure adjustment under these conditions it is desirable to use an empirically calibrated scale, which may be attached to some convenient place on or near the stand holding the apparatus.

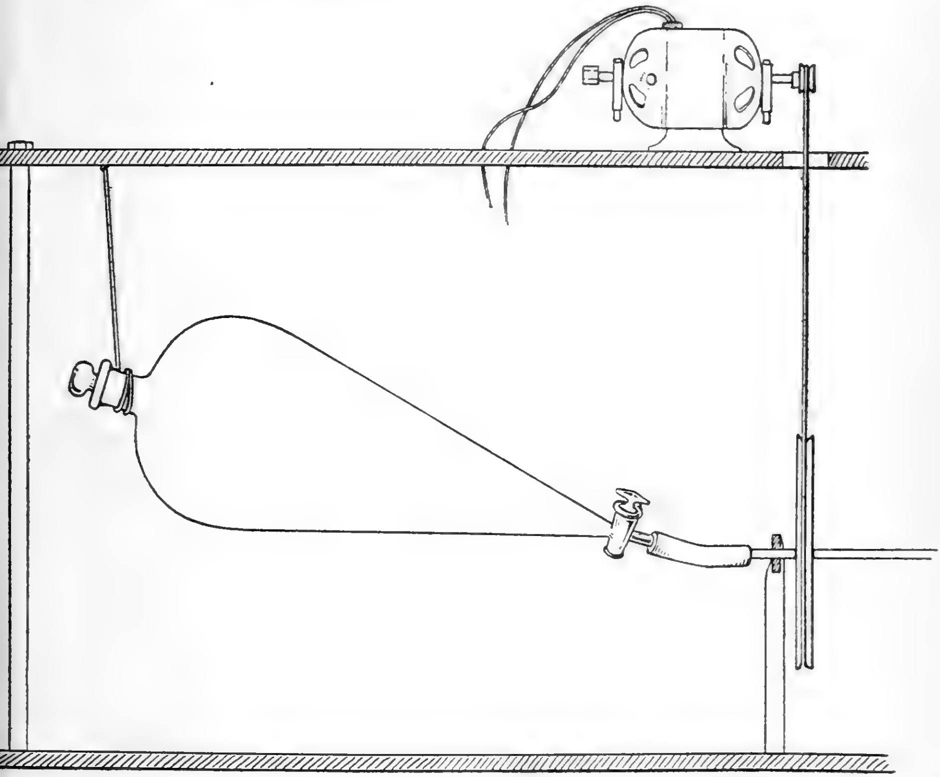

Fig. 2.

The scale is calibrated by placing in the chamber of the gas apparatus an amount of water equal to the final volume of the reaction mixture to be used (*e.g.*, 8 cc. for oxygen analysis of 2 cc. of blood). Then, with the upper cock of the apparatus open, to establish atmospheric pressure within, one marks on the scale the levels at which the surface of the mercury in the levelling

bulb is held when 0.10, 0.20, 0.30, etc., cc. of gas are present in the apparatus. In subsequent analyses the levelling bulb is adjusted by comparison with the scale thus prepared. For particularly careful adjustment, the levelling bulb may have an upright side arm of 6 or 8 mm. diameter sealed into it; the mercury surface in the arm can be levelled against the scale somewhat more readily than the broader surface in the bulb itself.[1]

Tonometer Rotator.—When a separatory funnel is used as a tonometer for saturation of blood with gas, the funnel may be conveniently rotated by the device shown in Fig. 2. The stem of the funnel is slipped into a rubber tube which fits over the axle of a pulley turned by a motor. To secure even distribution of the blood about the inner wall, the separatory funnel is so arranged that its lower side is horizontal. The proper position may be attained either by suspending the neck of the funnel in a wire loop, or by laying the funnel in a horizontal trough.

[1] Fig. 1 in the accompanying paper by Van Slyke and Stadie shows an apparatus provided with a levelling scale, and a levelling bulb with side arm.

THE CHEMICAL COMPOSITION OF THE OVARIES OF FRESH WATER GAR, LEPIDOSTEUS.*

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INTRODUCTION.

The data herein reported were obtained in the summer of 1917 as a part of the results of a study of fresh water fishery products undertaken with a view to extending the food sources available as meat substitutes. Gar eggs are of similar size and color to sturgeon eggs and have been unsuccessfully used as adultering substitutes for sturgeon eggs for caviar. The gar eggs do not retain their color in caviar processing and are not pleasing, in fact are positively objectionable, in flavor.

Chemical analyses have not previously been reported for gar roe. In fact there is a dearth of analyses of either roe or of ovaries of American fishes. Greene (1921) has analyzed the ovaries of the king salmon, and there is a single analysis of shad roe in Atwater's paper (1888.) Greene has followed the development of the salmon ovaries which occurs during the spawning migration. There is a great increase in egg mass with corresponding accumulation of protein and lipoids in the egg yolk, of which he presents evidence to show is derived from a corresponding storage in the muscles.

* Published by permission of the United States Commissioner of Fish and Fisheries.

† The data of this paper were used in part in the dissertation presented by Mr. Erwin E. Nelson for the degree of Doctor of Philosophy, University of Missouri, 1920.

University Fellow in the Graduate School, University of Missouri, 1916–18.

In Europe a number of workers, chiefly German, have studied the composition of roe and caviar. The earlier literature is given in Atwater's monograph on the food fishes of America (1888). More recent papers are those of Buttenberg (1900-02), Rimini (1904), Farnstein (1903-04), Albu and Neuberg (1906), and Weitzel (1916). Tangl and Farkas (1904) followed the changes in composition of the fish egg with development of the embryo. Solberg (1906) analyzed codfish roe. König and Grossfeld (1913) made a very complete study of the use of fish roe as food, taking up in considerable detail the proteins, fats, and extractives.

Material and Methods.

Our samples of gar ovary were obtained from twelve specimens of *Lepidosteus platystomus* and one sample of *Lepidosteus osseus.* We did not secure samples of the larger southern species, the alligator gar, *Lepidosteus tristœchus.*

The method followed is essentially that perfected by Janney (1916) and used by C. H. Greene (1919) in his study of the extractives of the muscle of the king salmon. The samples of tissue for analysis were in all cases taken fresh, generally while still physiologically alive. Samples of the ovaries were placed in weighed glass-stoppered bottles, weighed at once, and transferred to casseroles and extraction with hot alcohol was begun at once, or they were covered with 95 per cent alcohol and sealed with hard paraffin for transportation.

Samples for the determination of water were taken at the same time and weighed at once, then dried to constant weight at 105°C. Water determinations were generally made in duplicate.

The analysis of the samples was carried out in the following manner: the sample and the alcohol covering it were transferred quantitatively to a porcelain casserole of about 300 cc. capacity. The alcohol was brought to boiling to complete the coagulation of the proteins. It was then poured off into a wide mouthed short necked flask, and placed on the water bath. Subsequent alcohol and water extracts were placed in this flask, which was kept on the water bath. If the sample were very fat, two or three preliminary extractions with ether were made. The sample was next extracted with boiling water in 50 to 100 cc. portions, at least eight extractions being made. The residue was then

washed with 95 per cent alcohol and transferred to a Gooch crucible. The crucible was placed in a Greene's (1909) modified Soxhlet apparatus and extracted continuously with 95 per cent alcohol for 12 hours. This was followed with absolute alcohol for 12 hours, and then ether for from 18 to 24 hours. After the alcohol-ether extraction was complete the residue was transferred to a weighing vial, dried to constant weight at 105°C., and weighed. This fraction appears in the tables as the protein residue.

The preliminary ether extract, the ether from the Soxhlet, and the ether-soluble material obtained from the alcohol-water extracts as described below, were evaporated and dried at 50°C. and 75 mm. of mercury in a vacuum oven. The oily material was dissolved in dry ether, filtered into weighing vials, and dried to constant weight in the vacuum oven. This is the lipoid fraction of the tables. At this point there was generally a small amount of material which was insoluble in the ether. This was dissolved in water and added to the alcohol-water extracts as described below.

The alcohol and water extracts were combined and evaporated to dryness on the water bath. The dry residue was then extracted with ether, and the ether-soluble material added to that from the preliminary ether extraction and the Soxhlet extraction as mentioned in the preceding paragraph. The residue insoluble in ether was taken up with water and made up to 250 cc. This solution contains the extractives. 100 cc. were evaporated to dryness in a platinum shell, weighed, ashed, and weighed again. The water-soluble solids, and the ash of the water-soluble solids were obtained from these figures.

Total nitrogen of the extractives was determined in an aliquot by Gulick's modification of the Folin-Farmer colorimetric method (1914).

Creatine was determined in 10 cc. aliquots by dehydrolysis and calculated as creatinine (Folin, 1914). The 10 cc. samples were evaporated to dryness on the water bath with 10 cc. of N HCl and a bit of metallic lead, and the residue taken up quantitatively with hot water and washed into a 25 cc. volumetric flask. 10 cc. of saturated picric acid and 1 cc. of 10 per cent NaOH were added, the mixture was cooled at the tap, and allowed to stand. The necessary amount of standard creatinine solution

was placed in a similar flask and treated with NaOH and picric acid in the same manner. At the end of 10 minutes both flasks were filled to the mark with distilled water and the readings taken in a Duboscq colorimeter. All determinations were made by daylight. The standard creatinine was isolated from urine by Benedict's method (1914) and checked against a sample of pure creatinine from Dr. Myers' laboratory. The values as recorded in the tables of this paper are in terms of creatine obtained by multiplying the creatinine determination by the factor 1.16.

The amino nitrogen of the extractives was obtained by the method of Van Slyke (1912) using the micro-apparatus.[1]

Protocols with Descriptive Data for Chemical Samples.

Sample C11.—Fish 18. *Lepidosteus platystomus* ovary. Length of fish 52 cm., weight 385 gm. Weight of ovaries 15.9 gm., diameter of ova 1.2 mm. Weight of chemical sample 11.908 gm. Fairport, Iowa, July 25, 1917.

Sample C20.—Fish 19. *Lepidosteus platystomus* ovary. Length of fish 52 cm., weight 435 gm. Weight of ovaries 30.5 gm., diameter of ova varied to a marked degree. "Many small white immature ova." Largest ova 2.2 mm., smallest 0.8 mm. Weight of chemical sample 18.464 gm. Fairport, Iowa, July 25, 1917.

Sample C22.—Fish 32. *Lepidosteus platystomus* ovary. Length of fish 56.3 cm., weight 535 gm. Weight of ovaries 48.3 gm., diameter of ova 1.7 mm. Weight of chemical sample 17.858 gm. New Boston, Illinois, August 25, 1917.

Sample C26.—Fish 29. *Lepidosteus platystomus* ovary. Ovary weight 27 gm., diameter of ova 1.5 mm. "Ovary filled with red fat." Weight of chemical sample 20.958 gm. Fairport, Iowa, August 16, 1917.

Sample C29.—Fish 35. *Lepidosteus platystomus* ovary. Length of fish 57.5 cm., weight 504 gm. Weight of ovaries 48.5 gm., diameter of ova 1.9 mm. Weight of chemical sample 20.808 gm. New Boston, Illinois, August 25, 1917.

Sample C36.—Fish 33. *Lepidosteus platystomus* ovary. Length of fish 58 cm., weight 611 gm. Weight of ovaries 52.8 gm., diameter of ova 1.8 mm. Weight of chemical sample 20.373 gm. New Boston, Illinois, August 25, 1917.

Sample C38.—Fish 34. *Lepidosteus platystomus* ovary. Length of fish 55.4 cm., weight 619 gm. Weight of ovaries 72.5 gm., diameter of ova 2.0 mm. Weight of chemical sample 20.059 gm. New Boston, Illinois, August 25, 1917.

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[1] All total nitrogen and amino nitrogen determinations were made for us by Mr. Louis Gambee.

Sample 41b.—Lepidosteus platystomus ovary. Mixed sample. "In salt, 1 to 6. Roe immature, full of fat." Weight of chemical sample 20.071 gm. Received by express from New Boston, Illinois, September 26, 1917.

Sample 42a.—Lepidosteus osseus ovary. Weight of sample 22.958 gm. Weight of ovary estimated 500 gm. Received from New Boston, Illinois, by express November 12, 1917.

TABLE I.

*Summary of Results of Analyses of Gar Ovaries.**

No.	Lipoid.	Protein.	Extractives.					Water, by	
			Solids.	Ash.	Total N.	Amino N.	Creatine.	Difference.	Determination.
C11	11.3	15.7	2.08	0.56	0.217	0.056	0.0101	70.9	72.9
C20	14.0	23.8	2.75	0.49	0.218	0.049	0.0111	59.4	57.1
C22	12.4	26.3	1.13	0.31	0.291	0.028	‡	60.1	61.0
C26	20.6†	23.3	1.27	0.28	0.130	0.038	0.0069	61.5	56.2
C29	17.4	25.6	1.09	0.27	0.077	0.023	0.0085	55.8	57.0
C36	14.4	27.9	1.21	0.54	0.074	0.017	0.0051	56.4	57.1
C38	17.9	24.8	1.21	0.25	0.076	0.044	0.0080	56.0	56.4
41a	14.0	26.4	18.45	14.66	0.243	0.031	0.0082	41.1	‡
41b	12.8	26.7	17.16	10.82	0.229	0.036	0.0097	43.3	‡
42a	26.9	28.4	0.79	0.25	0.053	0.020	Trace.	43.9	‡
C63	17.3	26.3	1.15	0.51	0.091	0.015	‡	55.2	‡
C66	17.6	25.3	1.02	0.38	0.072	Trace.	‡	56.0	‡
C78	18.3	26.2	1.54	0.56	0.138	0.006	‡	54.0	‡

* No. 42a is from *Lepidosteus osseus*, the remainder are *Lepidosteus platystomus*. All calculations are in terms of parts per 100 gm. of moist sample.

† Part lost.

‡ Not determined.

Sample C63.—Lepidosteus platystomus ovary. Length of fish 61 cm., weight of ovary 66.7 gm. Weight of chemical sample 24.095 gm. Hannibal, Missouri, July 30, 1920.

Sample C66.—Lepidosteus platystomus ovary. Length of fish 58.5 cm., weight of ovary 91.65 gm. Weight of chemical sample 27.46 gm. Hannibal, Missouri, July 30, 1920.

Sample C78.—Lepidosteus platystomus ovary. Length of fish 55.8 cm., weight of ovary 94.75 gm. Weight of chemical sample 28.28 gm. Hannibal, Missouri, July 30, 1920.

DISCUSSION.

Lipoids.

The lipoid fraction in terms of the moist sample of ovary is comparatively large, from 15 to 20 per cent. In the single sample of *Lepidosteus osseus*, 42a, it reaches 26 per cent, which is higher than any value for fish ovary found in the literature. In the gar ovary, especially in the young stages, there is a deposit of fat in the supporting tissues which in some samples is quite considerable.

TABLE II.

Analyses Arranged in Series According to the Weights of the Ovaries.

No.	Weight of ovary.	Diameter of ova.	Length of fish.	Lipoid.	Protein.	Extractives.	Water.
	gm.	*mm.*	*cm.*				
C11	15.9	1.2	38.5	11.3	15.7	2.07	72.9
C26	27.0	1.5	*	20.6†	23.3	1.26	56.2
C20	30.5	2.2 to 0.8	43.5	14.0	23.8	2.70	57.0
C22	48.0	1.7	53.5	12.4	26.3	1.13	61.0
C29	48.5	1.9	60.4	17.4	25.6	1.09	57.0
C36	52.0	1.8	61.1	14.4	27.8	1.20	57.1
C63	66.7	*	61.0	17.3	26.3	1.15	53.1
C38	72.0	2.0	61.9	17.9	24.8	1.21	56.4
C66	91.6	*	58.5	17.6	25.3	1.03	56.0
C78	94.7	*	55.8	18.3	26.2	1.54	53.9
42a	‡	‡	‡	26.9	28.4	0.79	43.9

* Not recorded.

† The notes record that there was a large amount of extra-ovular fat in this ovary

‡ Not recorded, but this was undoubtedly the oldest fish of the series. The ovary weighed several hundred grams.

The analysis of the entire ovary does not distinguish between this fat, which might be called extra-ovular, and the lipoids of the developing ovules with their increasing mass of cell yolk. The total egg yolk lipoids seem to increase as the eggs develop.

If one arranges the analyses in a series according to the weight of the ovaries, it is noted that in a general way the percentage of ether-soluble materials increases with the increase in weight, Table II. The parallelism between increase in weight, diameter of the ova, and length of the fish shown in this table is evidence that the increase in lipoids is in part at least due to growth of

the ovary, and not to the accumulation of extra-ovular fat. The weight of the ovary is a fair criterion of the age of the ovary.

Because of the inability to differentiate between the source of the various lipoid fractions, it would seem that a more accurate picture of the metabolic changes might be gained by calculating the data on a fat-free basis. This has been done in Table III.

Proteins.

There is a rather close approximation in the values for the proteins, if Sample C11 and the single sample of *Lepidosteus osseus*, 42a, be disregarded. C11 is a very much younger fish, if one may judge by the weight of the ovaries and the diameter of the ova. Greene (1918) showed for the salmon that the protein content of the ovary was remarkably constant throughout the period of late development. Sample C11 is an interesting case of an ovary which has not yet reached the average protein content for the species. Its water content is the highest of all the specimens examined. Sample 42a is obviously a much more mature specimen. It has the highest protein, 28.4 per cent, and the lowest water, 43.9 per cent, content of all the ovaries studied. This might be explained on the grounds of the difference in species. But Hatai (1917) has shown that in the white rat there is an increase in protein and a decrease in water content of the whole body throughout the whole growth period. These facts, especially when taken in conjunction with the variations in the extractive fractions, argue for a more active stage of metabolism in the early growth period of the gar ovaries.

The average values for protein are in close agreement with those found by Greene (1918) for the salmon. They also agree closely with unpublished data for the ovaries of the carp.

Organic Extractives.

The organic extractives present in the tissue waters of all animal organs are an indirect measure of the metabolic processes in the individual tissue. Hatai (1917) has shown that the tissues of the growing white rat contain a greater proportion of extractives than those of the adult. During the migration of the king salmon Greene (1918) showed that the organic extractives

of the muscle at first increase slightly, then remain comparatively constant in proportion to the tissue waters until late in the spawning. At the spawning when the animals are approaching death by inanition the tissue waters are less saturated. In the developing ovaries the organic extractives are of a much lower concentration than in the muscles. The percentage does not vary much during the entire migration. C. H. Greene (1919) found with respect to the muscular tissue that while the absolute amount of protein decreases, the organic extractives, especially the amino nitrogen, remain constant or increase in spite of the fact that the protein from which they are derived is constantly decreasing. The lower concentration in the ovaries would seem to be a function of the anabolic processes whereby the ovarian extractives are being synthesized into proteins. At any rate the gar muscle extractives average about 2.5 per cent while the extractives of the ovaries average about 1 per cent.

If the ratio of organic extractives to protein be figured for the series given in Table II, it will be seen that the extractives exist in the largest ratio in the youngest ovary, C11 (Table III). In C20, which contained a large number of small immature ova, the ratio is also 1:10. In 42a, which was certainly the oldest specimen, the ratio is the smallest, 1:52. The other ratios vary in no regular order from 1:24 to 1:42. In the case of the amino nitrogen, the amounts are again certainly greatest in the youngest specimens, decreasing with maturity.

In general there is a good deal of variation in the figures for the fractions of the organic extractives; *viz.*, total nitrogen, amino nitrogen, and creatine. In view of the relation between amino nitrogen recently pointed out by C. H. Greene (1919) it is of interest to note that the sample having the highest water content, C11, has also the highest value for amino nitrogen, and that the one with the lowest water, 42a, gave with one exception the lowest value for amino nitrogen. The low value for C36 is not explained.

König and Grossfeld (1913) have studied the composition of the extractives of fish eggs, and have isolated xanthine, hypoxanthine, creatinine, taurine, and tyrosine. In view of the ease with which creatine is changed to creatinine in the manipulations of the analysis, Grindley and Woods (1906), it would seem doubtful whether there is really any preformed creatinine in fish eggs.

Creatine was found in gar eggs in amounts varying from traces up to slightly less than 10 mg. per 100 gm. of moist sample. No especial significance is attached to this observation.

The Inorganic Ash and Water.

Our data show variations of the ash from 0.25 to 0.59 per cent, a rather extreme variation, for which there is no obvious explanation.

Water determinations were made directly on separate samples of the first seven fish but only indirectly on the others. The water determination by differences throws the cumulative error on this fraction. Nevertheless, the agreement is quite close as between the indirect and the direct determinations on the first seven specimens.

TABLE III.

The Protein, Water, and Extractive Fractions Figured on a Fat-Free Basis, and Arranged in Series According to the Weights of the Ovaries.

No.	Weight of ovary.	Protein.	Organic extractives.*	Ratio protein: extractives.	Total N.	Amino N.	Water.
C11	15.9	17.7	1.70	10:1	0.244	0.063	79.9
C26	27.0	29.4	1.24	24:1	0.164	0.048	69.1
C20	30.5	27.7	2.63	10:1	0.254	0.057	69.1
C22	48.0	30.0	0.94	32:1	0.332	0.031	68.7
C29	48.5	31.0	1.00	31:1	0.092	0.027	67.6
C36	52.0	32.6	0.78	42:1	0.086	0.020	66.0
C63	66.7	31.8	0.66	48:1	0.111	0.018	64.6
C38	72.0	30.2	1.18	27:1	0.093	0.054	68.4
C66	91.6	30.8	0.79	35:1	0.088	Traces.	70.8
C78	94.7	32.0	1.19	27:1	0.168	0.007	66.0
42a	†	38.9	0.74	52:1	0.073	0.028	60.0

* The organic extractives were obtained by subtracting the ash from the water-soluble solids.

† Not recorded, but the weight was several hundred grams.

General Comparison.

We hoped to be able to compare the composition of the gar ovaries at different stages of development. However, these fish apparently spawn over a long season and there is no way of determining the degree of development except by the general appearance and the variation in size of the ovules. In our youngest specimen, C11,

the diameter is 1.2 mm. In C20, there was the greatest variation. A few ova were as small as 0.8 mm., while others were as much as 2.2 mm. in diameter. 2 mm. is the average diameter of the adult egg. In one very large fish, No. 42a, the total weight of the ovaries was estimated at 500 or 600 gm. These were the most mature in appearance of any collected. This ovary had a lower water content, the highest protein content, and a much higher amount of total fats. Broadly speaking, the protein except in the very young remains comparatively constant. The organic extractives are also constant. The total lipoids tend to increase and the total water to decrease with development of the gar ovaries.

We were not successful in securing ripe ova for analysis.

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THE CHEMICAL COMPOSITION OF THE SKELETAL MUSCLE OF THE FRESH WATER GAR, LEPIDOSTEUS.*

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INTRODUCTION.

The work herein reported was begun in connection with a study of the possible utilization of certain wastes from the commercial fisheries. The gar, *Lepidosteus*, is not ordinarily used as food, though it is quite plentiful in the Mississippi River and its tributaries. It is said to be used to some extent by the negroes in the South, and is seen occasionally in the fish markets of Baltimore, being purchased by the poorer class of Italians. Quantitative chemical analyses were made to determine the actual food value.

Historical.

There are no analyses of the muscle of the gar reported in the literature. The most important paper on the subject of fish muscle is Atwater's monograph on American food fish. This paper gives analyses of 123 specimens, belonging to 53 species. The literature up to 1888 is cited. Since that time a number of papers of various sorts have appeared, in which analyses of the flesh of fish are reported. Williams (1897) studied the composition of cooked fish. Paton (1898) in a series of valuable studies on the life history of the Scottish salmon, followed the changes in fat content through the period of development of the ovaries. He also studied the composition of the protein fraction of the

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muscles. Balland (1898) determined the composition of a number of fish with reference to their water content, fat, nitrogen, extractives, and ash. Lehmann (1900) determined that one can in some measure control the fat content of the flesh of fish by the diet. Lichtenfelt (1904) noted that the composition of the flesh of a given species varies considerably from time to time, and has attempted to follow the course of the variations. He explains them as due to the condition of hunger of the animal. König and Splittgerber (1909) determined the composition and food value of a number of European fishes. Suzuki and Joshimura (1909) studied the composition of the organic extractives of several European fish. Hollande (1913) gave data on the food value of certain exotic fish imported into France. Greene published a series of studies on the changes in the composition of the muscles of the salmon during the fast of the spawning migration (1913, 1915, 1916–17, 1919), in which quantitative analyses are given for the normal salmon as a basis for comparison of the changes occurring during the migration. Polimanti (1915) believed that the fish living near the surface of the sea are richer in fats than those having a deeper habitat. Clark and Almy (1918) have studied twenty of the American food fishes with especial reference to their seasonal variations. C. H. Greene (1918, 1919) has presented analyses of a second group of samples of the series of C. W. Greene on the salmon muscle. He confirmed the quantitative analyses and made a special study of the nitrogenous extractives of the muscle and their variation during the fast of the spawning migration.

Material and Methods.

There are two species of gar found commonly in the rivers of the United States, especially the Mississippi and its tributaries. These are the common or "billy" gar, *Lepidosteus platystomus* Linnæus, and the long-nosed gar, *Lepidosteus osseus* Rafinesque. In the South a third species, the alligator gar, *Lepidosteus tristœchus* (Block and Schneider) occurs, but no specimens of the species are included in these analyses.

The method followed is essentially that developed by C. H. Greene (1919) in his study of the extractives of the muscle of

the king salmon, and has been given in detail in the preceding paper.

The results of the chemical analyses are presented in Table I.

TABLE I.

*Analyses of the Muscle Tissue of Gar.**

No.	Source.	Lipoids.	Protein.	Organic extractives.	Ash.	Total N.	Amino N.	Creatine.	Water, by Difference.	Water, by Determination.
C27	*Lepidosteus platystomus.*	7.75	13.52	2.59	0.87	0.43	0.094	0.26	75.3	77.4
C31	*Lepidosteus platystomus.*	2.23	14.34	2.81	0.89	0.40	0.077	0.30	79.7	79.5
C37	*Lepidosteus platystomus.*	4.40	14.02	2.76	0.43	0.30	0.064	0.27	78.3	77.4
C35	*Lepidosteus osseus.*	6.39†	15.17	2.66	0.85	0.34	0.071	0.28	74.9	71.7
C41	*Lepidosteus osseus.*	7.75	14.64	3.17	0.92	0.51	0.091	0.26	73.5	71.7
42b	*Lepidosteus osseus.*	13.19	14.45	4.61	1.33	0.31	0.066	0.21	66.4	‡

* The calculations are in terms of parts per 100 gm. of moist sample.

† Part lost.

‡ Not determined.

DISCUSSION.

Lipoids.

The total lipoids (ether-soluble fats) of gar muscle vary from 2.2 to 13.19 per cent, Sample 42b being much the richest in lipoids. This sample came from a large specimen of *Lepidosteus osseus* from the Mississippi River at New Boston, Illinois, taken in November. All other samples are July and August fish which show relatively lower lipoid content. Clark and Almy (1918) have pointed out that the fat content of a number of the food fishes is highest in the autumn.

Protein.

The protein content of the muscles of the gar is uniformly about 14 per cent, the average of the determinations being 14.35 per cent. This figure is very much lower than that for the ovary of this species, the average protein content of which is 25 per cent. When one recalls the activity of the muscle, and the relatively passive condition of the ovary, this is a somewhat surprising observation. The figure is quite comparable to that for the muscles of the spawning salmon, 14 per cent, but is considerably less than that for the muscles of the normal salmon entering fresh water, 20 per cent figured on a fat-free basis. If a protein storage occurs in the gar, it is evidently on a much lower level than in the salmon.

Organic Extractives.

The organic extractives are a measure of tissue metabolism. This is indicated by the work of Hatai (1917), showing that the tissues of the growing white rat contain a greater proportion of extractives than those of the adult. Greene (1918) showed that during the period of migration of the king salmon, during which there is vigorous muscular activity, the organic extractives remain comparatively constant or actually increase, notwithstanding the decrease in the mass of muscle or in its diminishing percentage of protein.

The water-soluble solids and the organic extractives, Table I, are quite uniform if Sample 42b, which is again an extreme, be left out of account. The average for the total solids, 3.10 per cent, is quite comparable to that given by Greene (1919) for salmon muscle (average of fifteen samples 4.05 per cent).

The total non-protein nitrogen of the extractives varies from 0.30 to 0.51 per cent. Suzuki and Joshimura (1909) found for the tunny 1.09 per cent, a much larger value, and Buglia and Costantino (1912) report values of 0.47 and 0.57 per cent for certain teleosts of the Mediterranean.

The amino nitrogen is fairly constant, varying from 0.164 to 0.194 per cent. C. H. Greene (1919) has shown for salmon muscle that the fluids are an important factor in determining the amounts of amino nitrogen held in the tissues. For the salmon,

when the fluids present have a content of 100 mg. of amino nitrogen per 100 gm. of water, they act as though saturated, since the amino nitrogen shows no further change during the migration. When the figures in the present series are calculated per 100 gm. of water the following results are obtained:

Sample.	Amino N. *mg.*
C27	111
C37	82
C35	98
C41	126
C31	96
42b	100
Average	102

The average figure of 102 mg. is in interesting agreement with Greene's figures, though the variation is considerable.

Creatine varies from 0.21 to 0.30 per cent, or again leaving out of account Sample 42b, from 0.26 to 0.30 per cent. Myers and Fine (1913) have pointed out that the value for any species is constant and specific, as far as the adults are concerned. They observed that with kittens the value increased with age. If 42b was a sample from an older fish than the rest, as has been assumed through the paper, the determination of creatine should show a higher value than the rest, not a lower one. No explanation is offered for this discrepancy. Suzuki and Joshimura (1909) found the following values for creatine:

Fish.	Creatine. *per cent*
Bonito	0.100
Tunny	0.300
Salmon	0.320

Okuda (1912) reports values from 0.42 to 0.75 per cent in various teleosts. C. H. Greene (1919) considers Okuda's values too high.

In the original series of analyses creatinine was determined, but in view of the fact that Grindley and Woods (1906) deny the presence of creatinine in perfectly fresh muscle (confirmed by Mellanby, 1908), and show that merely evaporation of the watery extract of muscle on the water bath is sufficient to convert a large amount of the creatine into creatinine, the determinations have not been included in the tables. Suzuki and Joshimura

(1909) in experiments on the isolation of various substances from the extractives of fish muscle, did not isolate creatinine. Krukenberg (1881) who reports creatinine in fish muscle, was probably in error on this point.

Food Value of Gar Muscle.

The preceding analyses show that the gar flesh compares favorably with that of those species which contain the moderate amounts of stored lipoids.

The gar flesh contains from 2.23 to 13.19 per cent of total lipoids, an average of about 7 per cent. The caloric value of the total fats averages a slightly higher value than that of the total proteins.

The proteins average 14.37 per cent for the six samples and there is but little individual variation in the series. The gar flesh is on the whole a very palatable food of good caloric value.

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THE PROTEINS OF THE ALFALFA PLANT.*

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From a purely economic standpoint the green forage plant is the raw material from which all production is primarily derived. From a scientific standpoint it is of the greatest interest because in its leaves both carbohydrates and proteins are synthesized. Thus, the chemistry of the living plant presents problems of both practical and scientific importance. It is true that we know a multitude of products derived from plants and we know much of the chemistry of these, but this knowledge consists mostly of isolated facts which contribute comparatively little to a knowledge of the chemical make-up of the plant as a whole. Less is known of the chemistry of the proteins of the living plant than of any of its groups of constituents.

Rouelle[1] in 1773 announced that the glutinous matter, *i.e.* protein, which up to that time was known to exist only in the seeds of wheat, was present also in other parts of various plants. Later, he separated this glutinous substance from the juice of hemlock by heating to a moderate temperature and filtering out the coagulum, the protein nature of which was proved by the products of destructive distillation.

In 1789 Fourcroy[2] gave an extensive account of the occurrence of coagulable protein in the juices of various parts of many plants, and described the method by which he obtained prep-

*The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] Rouelle, *J. méd. chir., pharm.*, 1773, xxxix, 250.

[2] Fourcroy, A. F., *Ann. chim.*, 1789, iii, 252.

arations of what he supposed to be pure plant albumin. This observation of Fourcroy was the first to demonstrate the presence of two kinds of protein in plants.

A number of different investigators between 1799 and 1805 found albumin in the juices of many plants and in the sap of trees, but they did not add much to the information obtained by Fourcroy.

After 1805 no more attention was paid to the proteins of green plants presumably because, after Einhof[3] undertook his investigations of the seeds of cereals, the relative ease with which proteins could be obtained from seeds emphasized the difficulties of their extraction from green plants. It is a very remarkable fact, and not at all to the credit of science during all these years, that, apart from microchemical observations no attempt had been made to study the protein constituents of living plants until we undertook our investigation of the spinach plant.[4]

Various green forage plants contribute large amounts of protein to the ration of farm animals but practically nothing is known of the chemistry of these, even the proportion of protein in these plants not being yet established. It is true that the agricultural chemist states the percentages of protein in his analyses of green fodders, but these are made by indirect methods founded on assumptions unsupported by satisfactory evidence. A serious gap therefore exists in our current knowledge of the chemistry of nutrition which makes it impossible to apply to the practical problems of feeding on the farm what has been learned of the nutritive value of the proteins of the cereals as well as of the protein concentrates.

Special conditions are presented in dealing with the proteins of a living plant which deserve consideration.

First: the microscope shows that most of the protein is contained in cells, whose walls must be ruptured before the indiffusible proteins can be extracted.

Second: the living plant contains enzymes which rapidly convert one constituent into another and might be expected to profoundly alter the proteins before these could be extracted from the tissues and separated from solution.

[3] Einhof, H., *Neues allgem. J. Chem.*, 1805, v, 131; 1806, vi, 62.
[4] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1920, xlii, 1.

Third: the living plant contains a large number of constituents of many different types of known and unknown nature and these must be separated from the protein in order to obtain preparations of even roughly approximate purity.

The investigation reported in this paper embodies attempts to apply the methods developed in studying the proteins of the spinach leaf to alfalfa. Further experience with these methods has led to improvements, whereby it now appears to be possible to learn not only much that is new respecting the proteins of alfalfa but also respecting other constituents about which little is known.

By using suitable mills and presses relatively large quantities of the clear undiluted juice of the alfalfa plant were obtained, and also the water-soluble constituents were almost completely extracted within so short a time that autolytic changes were reduced to a minimum.

Chlorophyll, fats, phosphatides, etc., were removed by extracting with cold alcohol and ether, and so obtained uncontaminated with water-soluble substances which also might be soluble in these solvents.

The residue was then extracted at room temperature with dilute aqueous NaOH solution, but it was found that, although this solvent readily dissolves nearly all known types of protein, only a small part of the nitrogenous substances was thereby removed. Nearly all of the residual nitrogen was, however, extracted by boiling for a few minutes with 60 per cent alcohol containing 0.3 per cent NaOH.

Preliminary experience in developing these methods was gained during the course of the summer, so that it was late in the autumn before we were in a position to apply them satisfactorily on a relatively large scale. In order to continue this work during the winter alfalfa plants, cut about 3 months after sowing, were loosely packed in bags and placed in a cold room at −18°C. within 2 hours after cutting. The plants, thus quickly frozen, were kept at this low temperature until immediately before use. When removed from the cold storage room they appeared as fresh as when first cut. As their behavior during extraction was like that of freshly gathered plants, we believe that they had suffered no change sufficiently great to materially

affect the results of the experiments described in this paper. Since the results of the experiments with these frozen plants were more complete than those obtained with freshly gathered plants we describe them first in order that the reader may more easily understand our work.

The still frozen plants were put six times through the meat chopper and then the pasty mass was passed several times through a "Nixtamal" mill. The pulp, which weighed 22 kilos, was pressed in the hydraulic press, the press-cakes reground with 1,500 cc. of water and again pressed. This process was repeated. The washed cakes were then ground and kept under alcohol until the next day.

When the juice, obtained by directly pressing the ground plants, first ran out it was somewhat turbid and green from suspended chlorophyll grains. Later it ran free from chlorophyll and other suspended particles and was collected separately. This latter portion, which weighed 5,415 gm., represented the undiluted fluids of the plant. To this was added 18.7 per cent, by weight, of alcohol which produced a voluminous precipitate that was at once collected on large folded filters and allowed to drain out over night. The process thus far described was brought to this point within 6 hours.

The part of the press-juice which was green and turbid weighed 7,144 gm. This was filtered during the night through felts of paper pulp, whereby it was freed from chlorophyll and other suspended matters. The next morning there was a slight flocculent precipitate in the previously clear brown filtrate; this was removed by centrifuging. The treatment to which these two parts of the alfalfa juice were subsequently subjected will be described later.

The washed press-cakes, which had been brought into strong alcohol within 6 hours after the plants were first ground, were next extracted with about 9 liters of 93 per cent alcohol at room temperature three successive times and pressed out each time with the hydraulic press.

After practically all of the alcohol-soluble constituents were thus removed, the press-cakes were ground in the "Nixtamal" mill with a liberal quantity of 0.3 per cent NaOH solution and pressed in the hydraulic press. After thrice treating in the same way,

the press-cakes were boiled for about 5 minutes with 60 per cent alcohol, containing 0.3 per cent NaOH, drained on filters, and the residue pressed in the hydraulic press. After repeating this treatment the press-cakes were finally extracted with 93 per cent alcohol and dried in a current of warm air.

The green alfalfa plants were thus successively extracted with water, alcohol, dilute aqueous alkali, and with dilute alkaline alcohol. During these processes samples of extracts and residues were taken for analysis in order to determine the amount of solids, nitrogen, and inorganic matter removed at each stage. By allowing for the portions thus taken out we obtained the following data, which must be regarded as representing only the approximate proportions soluble in the different solvents.

	Ash-free solids.		Nitrogen.		Ash (CO_2-free).	
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
In the alfalfa taken	4,442		211.6		506.4	
Soluble in water	1,898	42.7	92.6	43.8	359.0	70.9
93 per cent alcohol	283	6.4	4.3	2.0	9.3	1.8
0.3 per cent aqueous NaOH	230	5.2	14.8	7.0	*	
0.3 per cent alcoholic NaOH	761	17.8	83.2	39.3		
Extracted residue	1,292	29.1	11.1	5.3		
	4,464	101.2	206.0	97.4		

* Satisfactory determinations of ash could not be made in the alkaline extracts, nor in the residue after these solvents were used.

We thus find that over 60 per cent of the ash-free solids and 83 per cent of the total nitrogen were extracted by water and hot alkaline alcohol. The small proportion of nitrogen soluble in aqueous alkali is striking, and shows the presence of relatively little nitrogenous substance insoluble in water and having the solubility in alkalies characteristic of most native proteins.

Although the hot alkaline alcohol dissolved only 17 per cent of the solids of the alfalfa plant, it extracted almost 40 per cent of the nitrogen, most, if not all, of which belongs to protein.

The small proportion of nitrogen left in the residue shows that the cells were ruptured so completely that nearly all of the nitrogenous substances were extracted by the various solvents.

The Water-Soluble Constituents of the Green Alfalfa Plant.

The brown press-juice, obtained as described on p. 66, may be considered to be free from suspended solids because the chlorophyll grains are so small and so difficult to separate by filtration that, if these are removed, as evidenced by the absence of any green deposit on centrifuging at high speed, appreciable quantities of other suspended matters cannot be present.

This brown press-juice contains a considerable amount of colloids which renders the solution opaque in thick layers. By transmitted light the juice is translucent and has a deep yellow color; by reflected light it is dark brown. The fresh juice is strongly acid to litmus, 19.0 cc. of 0.1 N NaOH solution being required to make 100 cc. approximately neutral to litmus and 13 cc. more to give a perceptible blue tint. At this reaction a considerable precipitate separates ($Ca_3P_2O_8$?).

The amount of solids contained in the juice undoubtedly varies with the state of development of the plant. One sample from plants gathered early in November, which had been sown August 19th, contained 9.26 gm. of solids per 100 cc. The juice of plants cut from the same field a few days later, and kept frozen for about 4 months, contained 10.52 gm. of solids per 100 cc. This somewhat higher content in solids is probably due, in part at least, to loss of water by evaporation during storage in the frozen state.

After standing for a few hours the clear brown juice slowly deposits a precipitate, which ultimately becomes considerable. A relatively large precipitate separates at once on slightly acidifying with acetic acid.

The "Colloid Precipitate."

As already stated, the colloids are precipitated by adding about 18 per cent by weight of alcohol. As the precipitate thus produced contains several substances of pronounced colloidal properties it will be hereafter referred to as the "colloid precipitate." Whether these colloidal constituents are chemically combined with one another, or are simply mixed together in the precipitate, has not been learned. The chief constituent is protein, but together with this is a considerable amount of calcium phosphate

and of organic calcium salts, including one, or more, coloring matters which have not yet been identified.

The colloid precipitate from about 12 liters of the clear brown juice of plants cut immediately before pressing was washed by centrifuging with water, 50 per cent alcohol, and finally with 74 per cent alcohol. Thus obtained in a highly hydrated state it was a compact gray-colored mass.

In order to remove calcium, phosphoric acid, and other substances soluble in acid alcohol, it was suspended in 5 liters of 74 per cent alcohol containing 0.1 per cent hydrochloric acid, centrifuged, and treated four times more, each time with 3 liters of the acid alcohol.

The united extracts, when made slightly alkaline to litmus with 1 per cent sodium hydroxide solution, yielded a large gelatinous precipitate resembling calcium phosphate, which will be referred to later as the "neutralization precipitate." This could not be removed by filtering, or by centrifuging, until heated to boiling. The clear, slightly alkaline filtrate and washings were then concentrated to 750 cc. *in vacuo* below 50° of the bath. Analysis of aliquots of this deep yellow-brown solution showed that the alkaline filtrate contained 20.66 gm. of solids, of which 16.3 gm. were NaCl, leaving only 4.36 gm. of other substances. It also contained 0.453 gm. of nitrogen. When this alkaline filtrate was made neutral to litmus with 0.1 N HCl, a turbidity formed which became a small flocculent precipitate on doubling the quantity of added acid. A part of the original solution when freed from alcohol and saturated with $(NH_4)_2SO_4$, gave a slight precipitate which, however, gave no biuret reaction. In view of this fact, and also of the small amount of nitrogen in this solution, it is evident that little, if any, protein had been removed from the "colloid precipitate" by the acid alcohol.

The results of this extraction in terms of dry solids were as follows:

	gm.	*per cent*
Insoluble in 74 per cent alcohol containing 0.1 per cent HCl	72.0	75.9
"Neutralization precipitate"	18.4	19.4
Filtrate from "neutralization precipitate"	4.5	4.7
Total	94.9	100.0

It thus appears that about one-fourth of the "colloid precipitate" was soluble in the dilute acid alcohol of which four-fifths was precipitated at a slightly alkaline reaction.

The nitrogen was distributed as follows:

	gm.	*per cent*
Insoluble in 74 per cent alcohol containing 0.1 per cent HCl	9.75	91.7
"Neutralization precipitate"	0.43	4.1
Filtrate from "neutralization precipitate"	0.45	4.2
Total	10.63	100.0

According to these figures the dry matter of the original "colloid precipitate" contained 11.2 per cent of nitrogen.

The ash was distributed as follows:

	gm.	*per cent*
Insoluble in 74 per cent alcohol containing 0.1 per cent HCl	1.10	9.8
"Neutralization precipitate"	9.82	87.5
Filtrate (estimated by difference)	0.30	2.7
Total	11.22	100.0

The original "colloid precipitate" accordingly contained 88.2 per cent of organic and 11.8 per cent of inorganic matter.

A portion of that part of the "colloid precipitate" which remained after extracting with the acid alcohol was washed with dilute and strong alcohol, dried at 107°, and analyzed with the following results:

Preparation 1.

	per cent
Ash	1.39
Ash-free	
Nitrogen	14.77
Phosphorus	0.18
Pentosans*	1.84

*Estimated from phloroglucide after distillation with HCl.

This preparation gave all of the characteristic color reactions of the proteins and, from the experiments later described, there can be very little doubt that nearly, if not all, of its nitrogen belonged to protein.

Another portion of the moist substance, equal to 6.15 gm. dried at 107°, was suspended in water and twice as much 0.1 N NaOH as was needed to impart an alkaline reaction was added to the suspension. This produced a thick viscid fluid which when centrifuged for some time at high speed, separated into a clear yellow layer at the top, gradually changing to a thick transparent jelly at the bottom, there being no line of separation at any point. The whole was poured into several volumes of absolute alcohol and 5 cc. of 20 per cent NaCl solution were added. The substance flocked out at once and then could be separated by centrifuging. After suspending the sediment twice more in absolute alcohol it was converted into a granular condition. Washed with ether and dried at 107° it weighed 5.77 gm., equal to 94 per cent of the substance taken. This preparation, No. 2, contained 8.18 per cent of ash and 15.85 per cent of nitrogen, calculated ash-free. This treatment with alkali and alcohol had, therefore, raised the nitrogen content by more than 1 per cent.

Another portion of this substance, equal to 16 gm. dried at 107°, was extracted five successive times by boiling each time for 1 hour with absolute alcohol, the insoluble part being separated by centrifuging. The first and second alcohol extracts contained only 0.0366 gm. of solids. After thus extracting the substance very thoroughly with boiling absolute alcohol, it was twice extracted by boiling with ether for 1 hour each time. This removed only 0.1868 gm. which, on drying in a beaker exposed to the air, formed a varnish-like film which did not redissolve in ether or in alcohol. The amount removed by ether was equal to only 1.1 per cent of the dry substance.

After thus freeing the "colloid precipitate" from everything soluble in water, acid alcohol, boiling absolute alcohol, and boiling ether, a portion was dried at 107°. This preparation, No. 3, contained 1.23 per cent of ash and, ash-free, 15.04 per cent of nitrogen, only a little more than before extracting with hot alcohol and ether. In order to further purify this substance by reprecipitation, 10 gm. were suspended in water and 0.1 N NaOH was added until the reaction was neutral to litmus, which required 100 cc. With 25 cc. more it became alkaline. After further adding 75 cc. of 0.1 N NaOH the mixture was warmed on the steam bath for a few minutes. On centrifuging a large part was deposited as a jelly, leaving the solution somewhat turbid.

The latter was decanted and the jelly mixed with 50 cc. of 0.1 N NaOH and heated for 1 hour on the steam bath. Under this treatment the jelly slowly dissolved. The clear solution was united with that obtained by first centrifuging, and then very slightly acidified by adding 0.1 N HCl until the precipitate separated sharply. In order to remove alcohol-soluble substances which might have been liberated by heating with alkali, this hydrated precipitate was suspended in alcohol, 35 cc. of 0.1 N NaOH were added, and the solution was reprecipitated by 28 cc. of 0.1 N HCl. The precipitate was first washed with 50 per cent, and then with strong alcohol, digested with absolute alcohol and finally with ether, and dried at 107°. This preparation, No. 4, contained 0.98 per cent of ash and, ash-free, 15.91 per cent of nitrogen. This figure is nearly 1 per cent higher than that found in the substance which had not been dissolved in hot alkali.

The filtrate and washings from this precipitate, both aqueous and alcoholic, were united, concentrated *in vacuo* to small volume, and subjected to a fractionation, the purpose being to learn the nature of the non-protein substances which it contained. Owing to the difficulties encountered in dealing with small quantities of substances of unknown nature nothing definite was learned beyond the fact that, apart from a relatively small proportion of protein and PO_4, the substances contained in these filtrates were chiefly coloring matters soluble in strong alcohol.

The outcome of this preliminary examination of the "colloid precipitate" shows that this is a mixture of substances of pronounced colloidal properties. In alcohol containing a little hydrochloric acid about 25 per cent of its dry solids are soluble, none of which is protein. Four-fifths of this part are precipitated by making the solution alkaline to litmus. This precipitate looks and behaves like calcium phosphate, which doubtless is its chief component, but together with this is a considerable quantity of one or more calcium salts of organic substances, as the following data show:

	gm.	*per cent*
Organic matter	8.62	46.8
Inorganic matter	9.82	53.2
Total	18.44	100.0

The ash contained 3.15 gm. of Ca and 2.56 gm. of PO_4, together equal to 5.71 gm., or to 58.1 per cent of the ash.

The PO_4 is equal to 4.18 gm. of $Ca_3P_2O_8$ containing 1.62 gm. of Ca, leaving 1.53 gm. of Ca equal to 3.82 gm. of $CaCO_3$. The sum of the calcium phosphate and carbonate is 8.00 gm., or 1.83 gm. less than the total ash. As the balance of this precipitate was used for other experiments these figures could not be confirmed, nor a direct determination of CO_2 be made; hence the nature of the unaccounted for balance of 1.83 gm. was not discovered.

From these data it appears that acid alcohol removes chiefly calcium salts of phosphoric acid and coloring substances which, in the free state, are very soluble in alcohol. When concentrated alcoholic solutions of the latter are poured into water colloidal solutions are formed, from which pigment is precipitated by adding a little NaCl. The sodium salt of the colored substance is very soluble in water with a deep orange-yellow color. Its tinctorial power is high. On adding acid, these yellow solutions become colorless at about the neutral point to litmus, and remain clear unless the solution contains relatively much of this substance.

What relation this coloring matter has to apparently similar substances heretofore obtained from vegetable sources we hope to determine in the future, but the properties above noted indicate flavone or flavone-like pigments.

Having thus found that the "colloid precipitate" contains a considerable amount of calcium salts of organic substances which in the free state are much more soluble in strong alcohol than in water, a relatively large quantity of another preparation of the "colloid precipitate" which had been washed thoroughly with 50 per cent and finally with 75 per cent alcohol was suspended in 2,000 cc. of water and centrifuged, in order to remove adhering alcohol. It was next suspended in 2,000 cc. of water containing 5 gm. of HCl and again centrifuged. The clear solution thus obtained gave no biuret reaction, nor any precipitate when a sample was saturated with $(NH_4)_2SO_4$, which shows that no protein had been dissolved by the acid.

When again extracted with 0.25 per cent aqueous HCl and centrifuged an opaque colloidal suspension resulted which could

not be decanted from the sediment. After adding about 60 gm. of NaCl and 5 gm. of HCl to the 4,000 cc. of solution and heating to 50°, a good separation was secured on centrifuging. The clear acid extracts were united, made faintly alkaline to litmus, heated on the steam bath for about half an hour, and then centrifuged.

When dried at 107° this precipitate contained:

	per cent
Loss on ignition	29.45
Ash	70.00
Nitrogen	0.55

The nitrogen was equivalent to only 1.84 per cent of the ash-free substance, indicating a relatively small proportion of nitrogenous substances in this "neutralization precipitate."

The ash contained:

	per cent
PO_4	18.94
Ca	36.04
Mg	0.78

These figures correspond to

	per cent
$Ca_3P_2O_8$	30.90
$CaCO_3$	60.15
MgO	1.30
Total	92.35

This "neutralization precipitate" consisted essentially of calcium salts of phosphoric acid and organic substances, the proportion of the latter being larger than was obtained from the extract made with acid alcohol.

The residue of the "colloidal precipitate," after thus extracting with dilute aqueous HCl, was suspended in water and shaken out with ether three successive times. The ether extracts were washed with water and evaporated *in vacuo.* The residue was taken up in absolute alcohol and likewise concentrated *in vacuo.* This process was repeated until water was removed. The residue was then extracted with absolute ether in which 0.5474 gm. was soluble. This is an almost insignificant proportion of the relatively large quantity of the "colloid precipitate" from which it

was derived. Unfortunately the dry weight of the portion taken was not determined, but was certainly more than 40 gm.

The residue of the "colloid precipitate" was next extracted three times with absolute alcohol. Owing to the colloidal condition of the solids it was difficult to obtain a good separation by centrifuging until a little NaCl was added. The 10,000 cc. of deep brown extract were concentrated *in vacuo* to 400 cc. and by centrifuging freed from a small amount of solids which separated during concentration. The clear solution was evaporated *in vacuo* and the residue extracted with absolute alcohol, in which most dissolved. Thus freed from sodium chloride, the clear alcoholic solution, when evaporated, left a very dark red-brown, amorphous residue weighing 3.32 gm. which was readily dissolved by 50 cc. of absolute alcohol. When this solution was poured into 150 cc. of distilled water a colloidal suspension resulted which did not separate on standing over night. The addition of a little HCl did not cause any separation, but the further addition of a little NaCl produced a large flocculent precipitate, leaving a clear, but deeply colored, solution. Examination of this pigment has, as yet, revealed no difference between it and the similar substances extracted by acid alcohol from the "colloid precipitate." It seems probable that this method of isolating the pigments may facilitate their further study.

After extracting the "colloid precipitate" with water, aqueous HCl, ether, and alcohol a portion was dried at 107° and analyzed with the following results.

Preparation 5.

	per cent
Ash	0.62
Ash-free substance.	
Total nitrogen	14.60
Amide "	0.97
Phosphorus	0.83
Sulfur	0.95

Another portion, equal to 13.5 gm. of the dry solids, was suspended in 950 cc. of water and 131.1 cc. of 0.1 N NaOH were added until the suspended solids separated sharply at the isoelectric point. The clear, almost colorless solution, when evaporated to dryness, left a residue weighing 0.7623 gm., consisting of NaCl. The NaOH added was equivalent to 0.7669 gm. of NaCl,

or to 0.4785 gm. of HCl, or to about 3.5 per cent of the dry solids of the preparation taken for this experiment. It thus appears that during extraction with HCl a notable amount of this acid had combined with the protein and that no water-soluble organic substance had been liberated at the isoelectric point.

In order to determine whether, or not, substances soluble in alcohol, but insoluble in water, had been liberated by thus removing the combined acid the solids were next suspended in absolute alcohol and centrifuged. The resulting clear solution, however, contained only 0.0620 gm. of solids, thus showing the absence of a notable quantity of substance soluble in alcohol, but insoluble in water, liberated at the isoelectric point.

To learn whether, or not, anything could be extracted by hot water the residue was next digested with water for an hour on the steam bath. After removing the suspended protein by centrifuging, the somewhat colloidal solution was treated with 8.5 cc. of 0.1 N NaOH and about 0.8 cc. of 20 per cent NaCl solution which caused the colloidally suspended matter (protein) to separate as a flocculent precipitate, leaving the solution clear when centrifuged. This precipitate, A, was treated as described below. The clear solution when evaporated left a residue, chiefly NaCl, weighing 0.2830 gm.

After thus extracting with hot water the residue was washed once by centrifuging with water. The washings contained only 0.1056 gm. The 0.3886 gm. thus extracted contained 0.1525 gm. of ash (NaCl) and 0.2361 gm. of organic matter. It is clear that only an insignificant amount of organic substance was thus extracted by hot water. The residue was next boiled with absolute alcohol and centrifuged. The alcoholic washings, on evaporation, left only 0.0276 gm. of solids.

The residue of Preparation 5, which had thus been freed from combined HCl and everything else soluble in hot or cold water as well as in alcohol, was dried at 107° and analyzed with the following results:

Preparation 6.

	per cent
Ash	0.74
Ash-free substance.	
Total nitrogen	15.45
Amide "	1.11
Phosphorus	0.081
Sulfur	1.02

The nitrogen content of this preparation was practically the same as that of Preparation 7, similarly obtained, which, ash-free, contained 15.34 per cent of nitrogen.

The small precipitate, A, p. 76, which separated from the hot water extracts after adding a little more alkali and some NaCl, as above noted, was dissolved in 20 cc. of 0.1 N NaOH, 100 cc. of water were added, and the solution was centrifuged. A part separated as a jelly from which the clear solution was decanted. To this solution 18.6 cc. of 0.1 N HCl were added, and the precipitate produced was washed with water and alcohol and dried at 107°. This preparation, No. 8, weighed 0.64 gm. and contained 0.63 per cent of ash. The ash-free substance contained 15.59 per cent of nitrogen; *i.e.*, only 0.24 per cent more than did Preparation 6 which had not been *dissolved* in alkali.

These data show that the residue of the "colloid precipitate," which remains after extracting with water, aqueous hydrochloric acid, ether, and alcohol, consists chiefly of a hydrochloride of protein which is entirely insoluble in water. Such unusual properties suggest combination with some non-protein group.

When this protein hydrochloride is suspended in water at the room temperature none is dissolved, as shown by the absence of a biuret reaction in the filtered solution. At temperatures approaching 100°, however, it is converted into a clear yellow jelly which does not pass into a true solution, even after long heating. After cooling, the addition of an excess of hydrochloric acid, or of sodium chloride, converts this jelly into a coarse flocculent precipitate resembling an ordinary protein coagulum, such as is commonly produced by heating. This settles rapidly, leaving the solution clear. A little coloring matter is thus removed, as shown by the strong yellow color of the filtered solution when made alkaline with NaOH.

When suspended in water and treated with NaOH to the isoelectric point the protein separates completely, leaving the solution water-clear. On evaporation a residue is left which is practically all sodium chloride. The combined hydrochloric acid is likewise converted into sodium chloride by sodium acetate.

A quantity of the moist protein hydrochloride, equal to 9 gm. dried at 107°, was suspended in water and freed from chloride by bringing to the isoelectric point with NaOH. The undissolved

protein was collected by centrifuging, suspended in 300 cc. of water saturated with toluene, and used for the following experiments.

Experiment I. 10 cc., containing 0.3 gm. of protein + 5 cc. of water.
Experiment II. 10 cc. + 3 cc. of water + 2 cc. of 0.1 N HCl.
Experiment III. 10 cc. + 5 cc. of 0.1 N HCl.

These mixtures, which contained 0.3 gm. of the protein, were heated simultaneously in a steam bath. Suspension III gelatinized first, Suspension II very soon after, and Suspension I appeared to be unchanged. After cooling Nos. II and III became firm jellies, which did not flow on inverting the test-tube, and on standing gradually contracted, leaving a little clear serum above. After 3 days there was much less serum in No. II than in No. III, suggesting that the acid was slowly hydrolyzing the protein, or possibly a combination of the protein with some non-protein complex.

Experiment IV. 10 cc. + 2 cc. of 10 per cent HCl + 3 cc. of water.

When heated on the steam bath the protein was precipitated like a heat coagulum, leaving the solution water-clear. This solution gave a strong biuret reaction showing the protein to be slightly soluble, perhaps owing to hydrolysis, when *heated* with acid of this strength.

After washing the precipitate with water it became slimy when the excess of acid was removed. When this was returned to the tube and heated as before, it formed a clear transparent jelly. On continued heating the jelly was slowly converted into a solution, which finally became so clear that it caused no Tyndall effect. This solution gave a flocculent precipitate with only 2 cc. of 0.1 N NaOH, which was completely redissolved by 1.2 cc. more. The ready solubility in dilute alkali, when contrasted with the behavior of this protein when treated with alkali before heating with acid, suggests that it exists as a conjugated protein which is hydrolyzed by the hot acid. See Experiment VI.

Experiment V. 10 cc. + 3 cc. of 20 per cent NaCl + 2 cc. of 0.1 N HCl.

Heated on the steam bath the effect was like that of No. IV, showing that the ions from NaCl prevent the formation of the

jelly as do those from HCl. After washing out the excess of salt and acid the protein was readily converted into a jelly when 5 cc. of 0.1 N HCl were added, and the mixture was heated on the steam bath. On further heating it behaved just like Suspension IV.

Experiment VI. 10 cc. + 15 cc. of water + 10 cc. of 0.1 N NaOH at room temperature.

This produced a viscid solution which was diluted to 100 cc. and centrifuged at high speed for sometime, whereby a small amount of gelatinous substance was deposited. Although the solution appeared to be nearly clear it filtered exceedingly slowly through paper and was quite opaque in a beam of sunlight. After heating in the steam bath for about 4 hours it could be readily filtered through paper.

A larger quantity of Preparation 5 was freed from combined HCl by adding NaOH to the isoelectric point and centrifuging. The deposit was centrifuged once with water and then suspended in 200 cc. of water and 25 cc. of 0.1 N NaOH were added. This produced a thick, opaque "solution," which was diluted to 400 cc. and centrifuged at high speed for some time. The nearly clear solution was decanted from a voluminous deposit of jelly and again centrifuged and decanted from a much smaller deposit. By suspending the jelly deposits in water and centrifuging repeatedly, as before, two parts were obtained, one representing the gelatinous fraction, the other the more soluble part. The solution of the latter, which could be filtered through paper fairly easily, was then precipitated by 17 cc. of 0.1 N HCl. This preparation, No. 9, was washed with water and with alcohol and dried at 107°. It contained 0.76 per cent of ash and, ash-free, 15.25 per cent of nitrogen. The jelly fraction was suspended in water and a little NaCl added. On centrifuging it separated as a coherent deposit which was washed with water and alcohol. Dried at 107° this preparation, No. 10, contained 2.05 per cent of ash, and, ash-free, 15.40 per cent of nitrogen. Although the solubilities of these two parts were so markedly different as to indicate the presence of two different proteins their nitrogen content was substantially the same, and also like that of the product from which they originated. Other data must be

obtained before the difference in solubility here noted can be explained.

Another sample of "colloid precipitate" was extracted four successive times with 93 per cent alcohol containing 0.1 per cent HCl and then treated with dilute sodium hydroxide, which produced a very turbid gelatinous "solution." This was centrifuged at high speed and the solution decanted from a jelly-like deposit. The latter was again suspended in dilute alkali and centrifuged, this process being repeated several times. The decanted solutions were united, filtered clear through paper pulp, and made neutral to litmus with dilute HCl. The resulting precipitate was washed with water, dilute and strong alcohol, and then with ether. Dried at 107° this preparation, No. 11, which weighed only 1.5 gm. and represented only a small fraction of the original substance, contained 1.4 per cent of ash and, ash-free, 15.33 per cent of nitrogen.

Having found the protein similarly obtained from spinach leaves to be readily soluble in *hot* dilute alkaline alcohol this solvent was applied to the alfalfa protein.

The leaves of freshly cut alfalfa plants were removed by hand and kept over night in the ice chest. The next morning 3,600 gm. of these were ground with 10 liters of water and then pressed in the hydraulic press. The green juice was filtered through paper pulp, which removed the chlorophyll, and the clear brown juice treated with about 20 per cent by weight of alcohol. The voluminous precipitate was washed, first with quite dilute and then with stronger alcohol, until adhering mother liquor was removed.

A part of this highly hydrated precipitate, equal to 19.7 gm. dried at 107°, was suspended in 1,000 cc. of 0.2 per cent NaOH solution, an equal volume of alcohol added and heated to 80° for 2 to 3 minutes. By centrifuging at high speed a yellow, gelatinous residue was removed which, when washed with alcohol and ether and dried at 107°, weighed 1.96 gm., and contained:

	per cent	
Ash	52.40	
Nitrogen	2.71	(= 5.69 per cent of the ash-free substance.)

The clear, alkaline filtrate when neutralized with HCl gave a flocculent precipitate, which was washed first with 50 per cent

and then with stronger and finally with absolute alcohol and dried at 107°. This preparation, No. 12, weighed 11.91 gm. and contained 0.62 per cent of ash. The ash-free substance contained:

Preparation 12.

	Protein.	Nitrogen.
	per cent	*per cent*
Ammonia nitrogen	0.96	5.86
Humin "	0.60	3.67
Basic "	3.76	22.98
Non-basic "	11.04	67.49
Total nitrogen	16.36	100.00

The percentage of nitrogen in Preparation 12 is about 1 per cent higher than that found in Preparations 8, 9, 10, and 11 which had been dissolved in aqueous alkali at *room temperature*. This fact indicates, that by the treatment with hot alkaline alcohol, some non-protein substance had been removed, possibly in consequence of hydrolytic action of the alkali. The following table gives the nitrogen found in the several fractions produced by the above described procedure.

	Grams.	N recovered.
		per cent
N in residue, insoluble in alkali	0.0600	2.7
N in alcoholic washings of above	0.0541	2.4
N in protein precipitated by HCl	1.8920	84.6
N in filtrate from above	0.2300	10.3
Total N	2.2361	100.0

These figures show that most of the nitrogen belongs to protein soluble in the hot alkaline alcohol and precipitable by acid.

Another preparation of the "colloid precipitate" was made in a similar way from plants cut a few days before blossoming. A portion of the moist preparation, containing 31.7 gm. of dry solids, 3.655 gm. of N = 11.54 per cent, and 3.552 gm. of ash = 11.12 per cent, was heated in the steam bath for a few minutes with 60 per cent alcohol containing 0.2 per cent of NaOH and

then centrifuged for some time at high speed. The gelatinous deposit was treated three successive times with hot dilute alkaline alcohol as before, and then washed with dilute and stronger alcohol. Dried at 107° this residue weighed 4.32 gm., equal to 14.3 per cent of the "colloid precipitate" from which it was derived. It contained 52.7 per cent of ash, equal to 68.1 per cent of the inorganic matter of the "colloid precipitate," and 2.51 per cent of nitrogen, equal to 3.1 per cent of the nitrogen in the "colloid precipitate." This nitrogen was equal to only 5.3 per cent of the organic matter in this preparation, indicating the presence of a large proportion of non-protein matter in this residue. *Cf.* similar product on p. 80.

The united alkaline solutions decanted from the foregoing residue were neutralized with HCl and the precipitate produced was washed with water, alcohol, and ether, and dried at 107°. This preparation weighed 17.72 gm., equal to 56 per cent of the "colloid precipitate," a considerably smaller proportion than was recovered in the precipitate in the corresponding experiment last described. The nitrogen content of this preparation, No. 13, was, however, practically the same as that of Preparation 12, being 16.21 per cent of the ash-free substance, as against 16.36 per cent.

The rest of the "colloid precipitate," from a part of which Preparation 13 was obtained, was worked up in the same way and yielded 233 gm. of Preparation 14 which contained 0.72 per cent of ash and 16.27 per cent of nitrogen, calculated to the ash-free substance.

50 gm. of the air-dried preparation, No. 14, equal to 44.68 gm., ash- and moisture-free, were boiled for 24 hours with 25 per cent of sulfuric acid and tyrosine, histidine, arginine, and lysine determined according to the slightly modified method of Kossel.[5]

Preparation 14.

	Protein.	
	per cent	*per cent*
Tyrosine	3.19	
Histidine	2.56	(containing N = 0.69)
Arginine	7.11	(" " = 2.29)
Lysine	3.34	(" " = 0.64)
Total N		3.62

[5] *Cf.* Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908, xxiii, 180.

The nitrogen contained in the basic amino-acids thus found agrees closely with the basic nitrogen found in Preparation 12, namely 3.76 per cent.

The Filtrate from the "Colloid Precipitate."

The filtrate from the precipitate produced by adding 18 per cent of alcohol to the alfalfa press-juice yields another voluminous precipitate when its alcohol content is raised to about 40 per cent by weight. This precipitate is a mixture of protein and inorganic salts, but as this treatment with alcohol renders nearly all of the latter insoluble in water or dilute salt solutions, it has been impossible, as yet, to learn anything of its characteristics.

We have, however, obtained evidence of a small proportion of heat coagulable protein, as well as of proteose, in the filtrate from the "colloid precipitate." Thus, 46 kilos of fresh alfalfa plant, equal to about 10 kilos dried, were thoroughly extracted with water and the clear extract was freed from the "colloid precipitate" by adding about 23 per cent by weight of alcohol. The filtered solution was then concentrated, heated to boiling, and acidified with acetic acid. The coagulum which separated was thoroughly washed with boiling water and then with alcohol and ether. Dried at 107°, this weighed 37.5 gm., and contained 43.0 per cent of ash. The ash-free substance, however, contained only 1.9 gm. of nitrogen, equal to 12.5 gm. of protein ($N \times 6.25$), or to only 0.12 per cent of the dry alfalfa solids, and this on the assumption that all of this nitrogen belongs to protein.

In another case 23 per cent of alcohol was added to the press-juice from alfalfa *leaves* containing 929 gm. of dry solids. The filtrate from the "colloid precipitate" thus produced was concentrated till the alcohol was removed, heated to boiling, and slightly acidified with acetic acid. The coagulum weighed 4.0 gm. when dried at 107°, and contained 0.4303 gm. of N and 0.5427 gm. of ash. The nitrogen in this coagulum was equal to 2.7 gm. of protein ($N \times 6.25$), or to 0.29 per cent of the dry solids of the leaves.

A part of the filtrate from this coagulum was saturated with $(NH_4)_2SO_4$ which produced an oily precipitate having the physical properties characteristic of proteoses. As this precipitate could

not be removed by an ordinary filter it was necessary to use a felt of paper pulp, which yielded a clear filtrate. The precipitate was redissolved by extracting the filter thoroughly with water, the filtrate again saturated with $(NH_4)_2SO_4$, and the oily precipitate treated as before. After twice more reprecipitating in this way the clear solution was made up to a definite volume, and total, as well as ammonia nitrogen determined in aliquots. The difference, 0.225 gm., was assumed to belong to proteoses equal to 1.406 gm. (N × 6.25). Since this was derived from 38 per cent of the total filtrate from the coagulum, this quantity is equivalent to 0.40 per cent of proteose possibly present in the solids of the dried leaves.

These figures, which must be accepted with great reserve, indicate that the alfalfa solids may possibly contain about 0.3 per cent of coagulable protein and 0.40 per cent of proteose, surprisingly small proportions to be found in such physiologically active tissues as are those of a green plant. By far the greater part of the protein of the juice of the alfalfa plant is, therefore, represented by the protein in the "colloid precipitate."

The Alcohol-Soluble Constituents of the Green Alfalfa Plant.

After extracting the 22 kilos of green alfalfa (see p. 66) with water the final press-cakes were ground in the Nixtamal mill with 8 liters of 93 per cent alcohol, pressed in the hydraulic press, and the cakes twice more treated in the same way with alcohol. These extracts were concentrated to relatively small volumes and solids and nitrogen were determined in aliquot parts of each.

	Solids.	Nitrogen.
	gm.	*gm.*
First extract	99.6	2.25
Second "	131.7	1.53
Third "	51.9	0.49
Total	283.2	4.27

Only 6.4 per cent of the solids of the dry alfalfa and 2.0 per cent of its nitrogen were thus extracted by alcohol.

The Alkali-Soluble Constituents of the Green Alfalfa Plant.

A part of the moist residue of the alfalfa plant, which had been extracted with water and then with alcohol, contained 482 gm. of dry solids, 20.58 gm. of nitrogen, and 22.55 gm. of ash (CO_2-free). This was ground with 5 liters of 0.3 per cent NaOH solution and pressed in the hydraulic press. The press-cake was again ground up with about 4 liters of 0.3 per cent NaOH and pressed as before. This process was repeated twice. The fourth extract contained scarcely anything precipitable by HCl.

The 18 liters of united extracts contained 2.70 gm. of nitrogen, 45.5 gm. of solids, and 3.5 gm. of ash (the two latter corrected for the added NaOH). These quantities are equivalent to 230 gm. of ash-free solids, 14.80 gm. of nitrogen, and 19.2 gm. of ash, calculated on the basis of the amount of alfalfa originally extracted. The nitrogen in this solution, therefore, was equal to only 7.0 per cent of the total alfalfa nitrogen, and the ash-free solids to 5.2 per cent of its organic matter. When acidified with dilute HCl this alkaline extract yielded a voluminous precipitate which was washed by centrifuging the dilute and stronger alcohol, and then extracted with absolute alcohol and ether.

This preparation, dried at 107°, weighed 13.5 gm., equal to 74 gm. from the entire lot of alfalfa plants originally extracted, or to 1.5 per cent of the dry matter thereof. It contained 0.55 gm. of ash and 1.305 gm. of nitrogen, equal to only 59 per cent of protein ($N \times 6.25$) in the precipitate. The ash-free substance, therefore, contained 9.37 per cent of nitrogen, equivalent to 7.15 gm., calculated back to the alfalfa taken, or to 3.4 per cent of the alfalfa nitrogen. When distilled with HCl this preparation yielded phloroglucide equivalent to 7.54 per cent of pentosan in the ash-free substance. Since this precipitate also gave strong xanthoprotein and biuret reactions there is little doubt that it consisted chiefly of a mixture of protein and carbohydrate.

Analysis of aliquot parts of the filtrate from the precipitate produced by HCl showed that it contained 32.5 gm. of solids, other than NaCl, 1.24 gm. of nitrogen, and 0.0953 gm. of ammonia, equivalent, respectively, to 178, 6.8, and 0.52 gm. calculated back to the amount of alfalfa originally taken. The ash-free solids were equal to 3.6 per cent of the dry alfalfa and the nitrogen

to 3.2 per cent of its total nitrogen. The ammonia in the filtrate from the acid precipitate was equal to 7.7 per cent of the total nitrogen extracted. Whether this ammonia resulted from hydrolysis of amide nitrogen of the protein, or from some non-protein nitrogenous substance, was not determined.

The remainder of the filtrate was saturated with $(NH_4)_2SO_4$, the precipitate produced filtered out and pressed on filter paper, redissolved, and again precipitated and pressed. When dissolved in a liter of water total nitrogen and ammonia nitrogen were determined in aliquots. The non-ammonia nitrogen thus found by difference, was equal to 0.4278 gm., or to 2.7 gm. of protein ($N \times 6.25$), in the entire solution. This quantity is equivalent to 14.7 gm. protein ($N \times 6.25$), or to 0.29 per cent in the alfalfa originally taken for extraction.

Extraction with Alkaline Alcohol.

After thus extracting with aqueous alkali, which might be expected to remove most of the residual protein, the press-cakes were ground up with 10 liters of 60 per cent alcohol containing 0.3 per cent NaOH, heated to boiling on the steam bath for a few minutes, and filtered hot. The residue was pressed in the hydraulic press and again treated in the same way.

The first extract was much more deeply colored than the second, and likewise yielded a much larger precipitate on the careful addition of dilute HCl.

The two precipitates were washed with 50 per cent and stronger alcohol, as well as with absolute alcohol and ether. That from the first extract weighed 54.2 gm., dried at 107°, and contained 1.37 gm. of ash and 7.63 gm. of N, equal to 14.42 per cent of the ash-free substance. The precipitate from the second extract weighed 12.0 gm., dried at 107°, and contained 1.15 gm. of ash and 1.17 gm. of nitrogen. The ash-free substance, therefore, contained only 10.74 per cent of nitrogen.

These two precipitates, together, contained nitrogen equivalent to 23 per cent of the alfalfa nitrogen, or to 6.09 per cent of protein ($N \times 6.25$), in the alfalfa plant.

The united filtrates from these precipitates contained 80.5 gm. of ash-free solids, equal to 8.9 per cent of the alfalfa; also 6.38 gm.

of nitrogen equal to 10.0 per cent of the ash-free solids, or to 16.5 per cent of the total nitrogen in the alfalfa.

Since heating with the alkaline alcohol might be expected to convert some of the amide nitrogen of the protein into ammonia, a part of the filtrate from the precipitate produced by adding HCl to the second alkaline alcohol extract was distilled with magnesia, and free ammonia equal to 2.2 per cent of the total nitrogen in this alkaline alcohol extract was found. This proportion of ammonia nitrogen was even less than that similarly found in the *cold* aqueous alkaline extract.

We thus find that alkaline alcohol extracted solids, equivalent to 16.0 per cent of the alfalfa, and nitrogen equal to 39.3 per cent of its total nitrogen.

The precipitate produced by neutralizing the first alkaline alcohol extract probably consisted chiefly of protein, as indicated by the 14.42 per cent of nitrogen in the ash-free substance, and by strong biuret and tryptophane reactions. It is almost certain that this crude precipitate contains some non-protein substance, probably mostly carbohydrate, but we have not, as yet, had an opportunity to determine this. We expect to subject this product to a critical examination and hope soon to be able to learn something of its relations to the protein of the "colloid precipitate" which it resembles in many ways.

The corresponding precipitate from the second alkaline alcohol extract contained, ash-free, only 10.7 per cent of nitrogen, and probably represents a still more impure preparation of the same protein.

The filtrate from the precipitate produced by HCl in the first alkaline alcohol extract was deeply colored and contained 52.8 gm. of ash-free solids, equal to 5.8 per cent of the entire lot of alfalfa extracted. This relatively large amount of the alfalfa solids had escaped extraction by water, strong alcohol, and aqueous alkali at room temperature. That it became soluble in water after heating with alkaline alcohol suggests that it was produced by hydrolysis during heating with the alkaline alcohol. Addition of neutral lead acetate to this filtrate gave a large precipitate containing much $PbCl_2$, together with a considerable quantity of organic lead salts. This precipitate was removed by centrifuging and decomposed with an excess of HCl. The addi-

tion of several volumes of absolute alcohol gave a deep yellow-brown solution which, after removing the $PbCl_2$, contained 23.3 gm. of solids (ash-free), largely soluble in absolute alcohol, and completely soluble in strong alcohol. This quantity is equal to 127.7 gm. from the total alfalfa originally extracted, or to 2.6 per cent. This represents only a part of the substances of this type, because the corresponding filtrate from the second extraction and the alcoholic washings of the precipitates, which were not treated with lead acetate, doubtless contained a further considerable quantity of the pigment. Since substances having similar properties, and resembling the pigments derived from flavone, were also obtained from the "colloid precipitate" it appears that the alfalfa plant may contain a very considerable proportion of this group of substances.

The fact that after the alfalfa had been previously extracted very thoroughly with water, alcohol, and then with aqueous alkali, heating with alkaline alcohol rendered so large an amount of the pigments soluble in water, as well as in alcohol, is strong evidence that both the protein and the pigment were derived from some combination, or combinations, hydrolyzed by the hot alkali. That simultaneously a correspondingly large proportion of protein was also rendered soluble in dilute aqueous alkali suggests that, in the plant, the protein and pigments may be combined. Such pigments may occur in green plants in much larger proportion than has heretofore been supposed, because apparently it has been overlooked that a large proportion of them may be combined with protein, as a complex insoluble in the solvents usually employed for extracting them. The methods employed in this investigation seem to be well suited for obtaining these pigments in a condition fit for further chemical study. We expect to continue our investigations along this line, and later we may be able to present results of interest to the chemist, as well as to the plant physiologist.

The Extracted Residue.

The residue from the extractions just described was nearly colorless and consisted chiefly of the fibrous structure of the plant. Dried at 107°, it contained 29.1 per cent of the dry, ash-free, solids of the alfalfa, but only 5.3 per cent of its nitrogen. In

view of the difficulties attendant on grinding large quantities of whole plants fine enough to rupture all of the cells so small an amount of undissolved nitrogen is surprising.

SUMMARY.

It is possible to grind the fresh green alfalfa plant so thoroughly that practically all of the contents of its cells subsequently can be extracted by water, alcohol, dilute aqueous alkali, and hot alkaline alcohol, applied in the order named.

Water extracts over 45 per cent of the dry matter of the plant, nearly 43 per cent of the ash-free solids, nearly 44 per cent of its nitrogen, and almost 71 per cent of its inorganic constituents.

By subjecting the ground plants to high pressure relatively large quantities of the undiluted juice of the plant can be obtained as an almost clear dark brown liquid, free from chlorophyll, or other suspended particles. This juice contains about 10 per cent of solids, a part of which is in colloidal solution. The addition of about 20 per cent of alcohol causes the latter to separate as a flocculent precipitate, which can be readily filtered out.

The filtered solution contains much nitrogen, but very little protein, probably less than 1 per cent. Some of this protein can be coagulated by heating the acidified solution, but more of it has properties characteristic of proteoses.

Most of the protein in the aqueous extract is in the precipitate produced by alcohol, which contains the substances previously in colloidal solution. In addition to protein, which forms upwards of 70 per cent of this precipitate, there are also present calcium phosphate and calcium salts of organic substances which can be extracted from the protein by alcohol containing HCl in which the protein is insoluble. The organic substances appear to be largely pigments which resemble the flavone derivatives already known to occur in many species of plants.

The protein combines with HCl without passing into solution at room temperature. When suspended in water this hydrochloride of the protein is converted into a jelly on heating, but does not dissolve, until heated for a long time with an excess of the acid. It behaves in a similar way toward dilute alkalies, but in this case passes into the gelatinous state at room temperatures.

Appreciable amounts do not dissolve, however, until the solution is heated. The precipitate of the colloids contains about 11.5 per cent of nitrogen. After extraction with 75 per cent alcohol containing a little hydrochloric acid the nitrogen content is raised to nearly 15 per cent. By suspending the protein hydrochloride, which results from the treatment with HCl, in water and adding dilute NaOH solution the protein separates at its isoelectric point in a flocculent condition. Practically nothing is thus removed except combined HCl. The nitrogen content of the protein is thus raised to nearly 15.5 per cent. When next treated with an excess of alkali at room temperature and precipitated by HCl the nitrogen content remains unchanged. No appreciable amount of substances soluble in alcohol, or in ether, are thus liberated by either the acid or alkali.

When dissolved by heating with an excess of dilute alkali and precipitated with HCl preparations containing about 16.3 per cent of nitrogen were obtained. The nature of the non-protein complex thus removed has not yet been determined, but the deep yellow-brown color of the alkaline solutions, and the yellow color developed by adding alkali to the filtrate from the precipitate produced by acid, indicate the presence of the same pigment found in the acid alcohol extracts of the mixed colloids. Since this pigment is not removed by treatment with alkali at room temperatures, but is removed by heating, it seems probable that the protein is combined with this pigment, from which it can be separated only by hydrolyzing the complex.

After removing the water-soluble constituents, 6.4 per cent of the alfalfa solids and 2.0 per cent of the nitrogen were obtained by extracting several times with alcohol. This extract contained nearly all of the chlorophyll, together with other substances, the nature of which has not yet been ascertained.

Dilute sodium hydroxide solution extracted only 6.9 per cent of the alfalfa nitrogen, a part of which was protein precipitable by slightly acidifying the extract. The precipitate also contained some pentosans.

After thus thoroughly extracting with water, alcohol, and dilute aqueous sodium hydroxide solution, the residue was boiled for a few minutes with 60 per cent alcohol containing 0.3 per cent NaOH. This treatment extracted 17.8 per cent of

the ash-free solids and 39.3 per cent of the nitrogen of the alfalfa. About 60 per cent of this nitrogen belongs to protein, which can be precipitated by the cautious addition of acid. Some, or all, of the remaining 40 per cent may have been derived from the same protein in consequence of changes caused by the hot alkali.

The protein in the residue of the alfalfa, after extraction with water and alcohol, behaves much like that found in the "colloid precipitate" produced by adding a little alcohol to the aqueous extract. That this, likewise, may be a complex containing protein and a flavone-like pigment is indicated by the liberation of a relatively large proportion of the latter simultaneously with the protein, when the alfalfa residue is heated with the alkaline alcohol.

After the successive extractions here described the residue was equal to 32 per cent of the solids of the plant, but contained only 5.6 per cent of its nitrogen. Consequently we can conclude that by the methods we have employed nearly all of the cells of the plant were ruptured so that their contents could be extracted by suitable solvents.

The procedure here outlined seems especially suited for separating the various groups of substances soluble in the several solvents under conditions particularly favorable for further investigation. We hope in the near future to be able to supplement this preliminary investigation with more detailed studies of these different groups.

THE USE OF SODIUM SULFATE AS THE GLOBULIN PRECIPITANT IN THE DETERMINATION OF PROTEINS IN BLOOD.

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In connection with a study of blood under normal and pathological conditions we proposed to use the method of Cullen and Van Slyke (1), for the determination of fibrin, globulin, and albumin nitrogen of blood plasma. A serious objection to the method is the use of ammonium sulfate as the globulin precipitant. This is so for two reasons, (*a*) because of the use of an ammonium salt which must be removed before determining the globulin nitrogen and (*b*) because of the physical difficulties involved in the removal of this nitrogen with magnesium oxide; particularly with reference to the "bumping" of the mixture. Both of these factors were realized by Cullen and Van Slyke, who, however, showed their method to be accurate, and believed that they had found a way of preventing the extreme bumping. We have corroborated their method as far as duplication of results is concerned but found that Merck's highest purity magnesium oxide is not always suitable for these determinations. Satisfactory results were obtained only by constant shaking of the Kjeldahl digestion flask during the distillation of the ammonia and the early part of the digestion.

The use of a non-nitrogen-containing precipitant in place of the ammonium sulfate, it seemed, would remedy both of the defects indicated above. The salt most commonly replacing ammonium sulfate for the precipitation of the total globulins is magnesium sulfate. Sodium sulfate has also been shown to be a satisfactory precipitant for globulin. Preliminary trials with these two salts indicated that, with regard to clean pre-

cipitation and rapid filtration, sodium sulfate was far superior to magnesium sulfate. When magnesium sulfate is used to precipitate proteins, the precipitates are gelatinous in character and the solutions filter slowly. The chief objection to the use of sodium sulfate is the necessity of working at temperatures above 34°C. for precipitation at the highest concentrations of the salt. The solubility of $Na_2SO_4 + 10\ H_2O$ increases gradually up to approximately 10°C. and then rapidly to 34°C.; above 34°C. the anhydrous salt is in equilibrium with water and the solubility of the salt decreases gradually. Working at incubator temperatures conditions with regard to solubility are those which obtain in general with magnesium sulfate and ammonium sulfate; *i.e.*, a *gradual* change in solubility with each increment of temperature.

The use of sodium sulfate for the precipitation of proteins is not a new procedure, nor is the use of it in the determination of the blood proteins a new process. Pinkus (2) realized the advantages of sodium sulfate in the study of proteins. He outlined the possibilities for its use and showed that (1) sodium sulfate possesses at 30°C. the same protein-precipitating power as ammonium sulfate. (2) When the anhydrous salt is used instead of the hydrated salt, at the temperature indicated, it precipitated globulins at the point of half saturation (about 25 per cent) and albumin at full saturation (about 50 per cent), it also allows of a fractionation of the proteins of Witte's peptone. (3) The use of sodium sulfate presents the following advantages: (*a*) the color reactions are hardly at all interfered with, (*b*) the nitrogen of the precipitate may be estimated directly according to method of Kjeldahl, (*c*) it is easy to obtain solutions containing little salt (5 per cent) by cooling, and (*d*) the salt itself is practically nontoxic. (4) By adding to protein solutions enough anhydrous sodium sulfate to absorb all of the water, a product is obtained which can be kept without change in the protein and is easily workable.

Porges and Spiro (3) confirmed the observation of Pinkus with regard to the precipitation limits of sodium sulfate: that the limits in terms of percentage of a saturated solution are approximately the same as those pertaining to ammonium sulfate. These authors used sodium sulfate in the quantitative separation of the serum proteins. They present evidence pointing

toward the presence of three globulins, a euglobulin and two pseudoglobulins, with precipitation limits at 28 to 36 per cent, 33 to 42 per cent, and 40 to 46 per cent of saturated sodium sulfate estimated from their published chart. Haslam (4) made use of sodium sulfate in the separation of albumoses.

Homer (5) has recently used sodium sulfate in the concentration of antitoxin and finds that antitoxin may be concentrated with sodium sulfate without denaturing by heat fully as well as with ammonium sulfate with heat. The removal of the salt is, moreover, simplified when sodium sulfate is used. Miss Homer constructed curves showing the percentage of protein precipitated by increasing amounts of ammonium sulfate and of sodium sulfate under various conditions of acidity and heat treatment and found them to be similar. She did not find critical points in the curves for the precipitation of eu- or pseudoglobulin or of serum albumin from undiluted serum. Mellanby (6) had previously failed to find critical zones with ammonium sulfate or magnesium sulfate. The following limits for the precipitation of the serum protein at 35–40°C. were found: pseudoglobulin precipitation complete at 11.5 to 18.5 per cent of anhydrous sodium sulfate, albumin completely precipitated at 32 per cent of anhydrous sodium sulfate. The precipitation of the individual proteins began at, and was complete at, lower concentrations of ammonium sulfate or of sodium sulfate than was required for their precipitation from their respective separate solutions in saline solution. There was also a greater overlapping of the precipitation limits for the individual proteins than was found for their separate solutions. The limits given hold for concentration of protein between 6.5 and 10 per cent. When the concentration of protein was reduced to 2.5 per cent, 34 gm. of sodium sulfate in 100 cc. were required for precipitation. Further dilutions required an increase in the concentration of the precipitating salt. The concentration of the electrolyte required for precipitation was affected by the reaction of the plasma, but a variation in reaction between pH 5.3 and 9.3 has only a slight effect on the result.

Spiro (7) has studied the effect of the addition of neutral salts upon the hydrogen ion concentration of protein solutions. The addition of either sodium sulfate or magnesium sulfate to a pro-

tein solution between the proportions of 1 part of salt to 9 parts of protein solution and 9 parts of salt and 1 of protein solution caused a change in the hydrogen ion concentration of the mixture from pH 7.73 to 7.38 for sodium sulfate and pH 7.73 to 7.27 for magnesium sulfate. On the other hand, the addition of ammonium sulfate causes a change of hydrogen ion concentration of from pH 7.73 to 5.91.

In the light of the results obtained by Miss Homer and also Kauder (8) and Hofmeister (9) with regard to the variation in the quantity of salt required for the precipitation of proteins it became necessary to determine the optimum concentration of sodium sulfate for the precipitation of the total globulins. Dilution of serum has experimental evidence in its favor; Porges and Spiro and particularly Wiener (10) have diluted the serum before precipitation. Wiener, using ammonium sulfate, came to the conclusion that the accuracy of his results was enhanced by dilution; he obtained a smaller quantity of globulin under such conditions. Contrary to the findings of previous workers we found a critical zone and indications of more than one critical zone by using concentrations of sodium sulfate which differed from the preceding member of the series by 1 per cent of the anhydrous salt.

Determination of the Critical Zone for the Precipitation of Total Globulins.—A series of 50 cc. portions of sodium sulfate at 37°C. containing increasing quantities of sodium sulfate was measured into or prepared in wide mouth glass-stoppered bottles. 5 cc. of blood plasma were added to each bottle and the bottles placed in the incubator at 37°C. After 3 to 12 hours in the incubator the solutions were filtered and an aliquot, 25 cc., of the filtrate was taken for analysis. Nitrogen was determined by the method of Kjeldahl.[1] The results for the most complete experiments are given in Chart 1 together with other data concerning the range of constant nitrogen values.

Later, in our desire to make use of sodium sulfate as the precipitant of protein in the determination of globulin when using small quantities of plasma or serum, it seemed desirable to work at higher dilutions. In this work two procedures were adopted:

[1] The addition of potassium sulfate is not necessary since sodium sulfate can take the place of potassium sulfate in the digestion.

CORRECTION.

n page 97, Vol. XLIX, No. 1, November, 1921, 4 lines from the top, *1:33* read *1:30;* 6 lines from the top, for *1:32.3* read *1:31.* Page 99, t-note 3, for *1:9.1* read *1:11.*

(*a*) The plasma was diluted 1:10 with 0.8 per cent sodium chloride solution and 5 cc. of the diluted plasma, equivalent to 0.5 cc. of plasma, were added to 10 cc. of the required concentration of sodium sulfate; this gave a dilution of 1:33. (*b*) To 15 cc. of a given solution of sodium sulfate 0.5 cc. of plasma was added which gave a dilution of 1:32.3. The results obtained with these two procedures indicated that there was not an essential increase in accuracy by diluting the plasma before adding it to the salt solution. In later work, therefore, the salt solution was added directly to the serum.

Evidence showing the effect of dilution upon the precipitation of blood proteins was obtained from three sources: (*a*) by coagulating the proteins of different samples of plasma at increasing concentrations of sodium sulfate (Chart 1), these data served to establish the probable critical zone of precipitation; (*b*) by precipitating the same sample of plasma over a short series of increasing concentrations of sodium sulfate which covered the probable critical zone and in increasing dilution (see Table I); and (*c*) by using a short series of concentrations of sodium sulfate with a number of different plasmas. Data from the last set of experiments are not included in this paper but appear in part in the succeeding one since they are to be considered only as confirmatory results and are of particular value in connection with the relative accuracy of the macro method and the micro method described there.

Data are presented in Chart 1 which indicate that at 37°C. a zone exists between 20 and 22 per cent[2] of anhydrous sodium sulfate in which an increase of 1 per cent of sodium sulfate does not cause an increase in the quantity of protein precipitated which is greater than the error of the method (a maximum analytical error of approximately 1.5 per cent). On either side of this zone a change of 1 per cent in the concentration of the salt gives a result which is distinctly greater or less than the results obtained at the critical zone.

The critical zone between 20 and 22 per cent of anhydrous sodium sulfate was indicated by the results obtained in our first work

[2] By 22 per cent of sodium sulfate we mean 22 gm. of sodium sulfate contained in 100 cc. of solution.

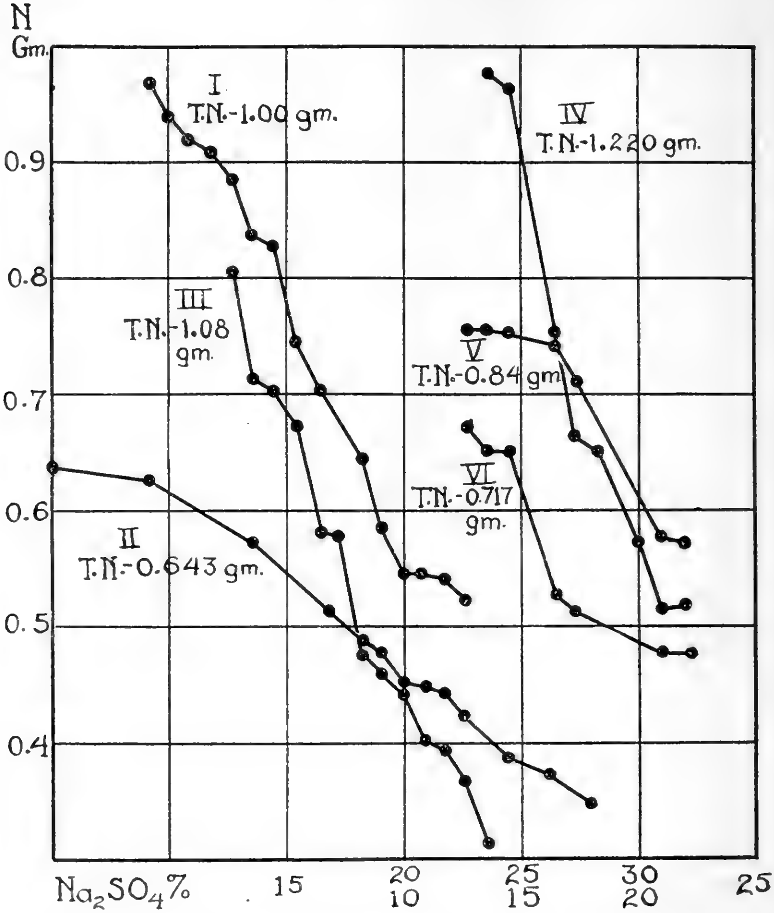

CHART 1. Curves showing the quantity of nitrogen, as grams of nitrogen per 100 cc. of serum or plasma, remaining in the filtrate after precipitation with increasing concentrations of sodium sulfate at 37°C. Curves I and II are for plasma and Curve III for serum at a dilution of 1:9.1. Curve IV is for plasma and Curves V and VI are for serum at a dilution of 1:32.3. The abscissæ are in terms of concentrations of anhydrous sodium sulfate expressed as per cent and the ordinates are expressed as grams of nitrogen in 100 cc. of blood.

at a dilution of 1:10.[3] These values do not hold absolutely for all degrees of dilution nor for all samples of blood as can be seen from Table I and Chart 1. With increasing dilution of the plasma there is a tendency for the critical zone to shift in the direction of a higher concentration of salt, 21 to 23 per cent of sodium sulfate. Furthermore, at the higher dilutions the amount of protein precipitated is less than at the lower dilutions. These facts have been noted previously for various salts by other investigators. The range of salt concentrations over which there is not a marked increase in precipitation for a small increment in salt may extend only over 2 per cent of sodium sulfate. On the

TABLE I.

Data indicating the effect of the dilution of blood serum or plasma upon the precipitation of total globulins by sodium sulfate at 37°C. Results are expressed as grams of nitrogen per 100 cc. of plasma and represent the quantity of nitrogen remaining in the filtrate after precipitation.

Na_2SO_4	Dilutions.				
	1:1	1:10	1:20	1:30	1:40
per cent					
19	0.499	0.511	0.516	0.536	0.553
20	0.486	0.487	0.492	0.518	0.516
21	0.459	0.428	0.411	0.443	0.437
22	0.437	0.416	0.393	0.430	0.439
23	0.380	0.383	0.387	0.412	0.393
24	0.357	0.354	0.336	0.399	0.369
25	0.352	0.315	0.303	0.341	0.343

other hand, a sharp break in the curve of precipitation with increasing salt concentration has always been observed at approximately the range of concentrations designated as the critical zone.

The variability of the critical zone with dilution and to a less extent with the sample of blood raises a question as to the value of the results and as to the concentration of sodium sulfate which will probably be in the critical zone if only one precipitation is adopted. Our observations have been in most cases on blood which is freshly drawn. In some cases determinations have

[3] As a convenience in measuring, 5 cc. of plasma were added to 50 cc. of salt solution which gave an actual dilution of 1:9.1.

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been repeated on old plasma and have shown that in many cases 4 to 6 days later results obtained upon precipitation of the globulin are identical with those obtained with fresh blood. The fact is stated in support of the critical zone but not in support of the practice of analyzing old plasma. From the data presented and from that obtained in the analyses of blood in which two or more concentrations of sodium sulfate have been used, the precipitation of blood plasma or serum in a solution which contains 21.5 gm. of anhydrous sodium sulfate per 100 cc. of solution, or 21.5 per cent, is most likely to correspond with the critical zone. At dilutions of plasma of 1:10 or higher the concentration given falls in the critical zone. The critical zone may be on either side of 21.5 per cent of the salt, but this concentration has been one of at least two concentrations in which the quantity of globulin precipitated is essentially equal. Ordinarily results obtained at 21 and 22 per cent of sodium sulfate fall in the critical zone.

Where it is desired to be assured of the presence of the critical zone determinations have been made at both 21 and 22 per cent of sodium sulfate. If both results at these concentrations agree it is assumed that the critical zone has been reached. If there is a marked difference in the results a precipitation is made at 23 per cent. In no case have we failed to obtain results in which one of the two pairs of concentrations showed an essentially constant degree of precipitation.

Ocular evidence has often been obtained of a change in the quantity of protein thrown down when the precipitation took place in test-tubes under which conditions the volume of precipitated protein shows a marked increase at the critical zone.

Comparisons of the protein precipitated by sodium sulfate at 21 to 22 per cent of sodium sulfate with that obtained with one-half saturated ammonium sulfate and magnesium sulfate at dilutions of 1:10 indicate that slightly less protein separates out when ammonium sulfate is used and more protein when magnesium sulfate is added. The results with magnesium sulfate have been repeatedly confirmed in other experiments. The data are contained in Table II.

Pinkus (2) gives 18.8 gm. of anhydrous sodium sulfate per 100 cc. as the point of complete precipitation of the globulin of ox serum $\frac{1}{4}$ dilute, horse serum $\frac{1}{15}$ dilute, serum globulin

7 per cent; and 20 gm. per 100 cc. solution for horse serum $\frac{1}{10}$ dilute and blood (ox) $\frac{1}{4}$ dilute. The results given above for serum diluted approximately 1:10 agree with that of Pinkus. On the basis of our results we feel justified in accepting the results obtained at 21.5 per cent of sodium sulfate as representing the precipitation of all globulins of blood serum or plasma.

The results of Porges and Spiro pointed to the possibility of other critical zones at approximately 12 to 14 per cent and at 18 per cent of sodium sulfate. Working with increasing concentrations of sodium sulfate we found critical zones at approximately the above concentrations (see Chart 1). These zones correspond roughly with the point of beginning precipitation in the chart

TABLE II.

Data relating to the quantity of nitrogen remaining in solution after precipitation with concentrations of sodium sulfate at the critical zone for total globulin and with one-half saturated ammonium sulfate and saturated magnesium sulfate at room temperature. Results are expressed as grams of nitrogen per 100 cc. of blood and represent the amount of nitrogen remaining in the filtrate after precipitation.

	Concentration.	Pig.	Goat.	Horse.
	per cent			
Sodium sulfate at 37°C............	21.3	0.393	0.399	0.443
	22.5	0.402	0.383	0.430
	23.2	0.311	0.372	0.412
Ammonium sulfate................		0.526	0.443	0.477
Magnesium "			0.323	0.385

of Porges and Spiro. They are not, however, always as definite as the critical zone at 21.5 per cent of sodium sulfate. The zone at 12 to 14 per cent of sodium sulfate would correspond to euglobulin which is usually precipitated by dilution and acidification with carbon dioxide or acetic acid and sometimes with saturated sodium chloride. The zone at 17.4 per cent of sodium sulfate would correspond to the second globulin of Porges and Spiro which is sometimes designated pseudoglobulin I.

Relation between the Quantity of Protein Precipitated at 13.4 Per Cent of Sodium Sulfate and with Sodium Chloride or Acidification with Carbon Dioxide.—It was assumed that the protein precipitated at the critical zone at about 13.4 per cent of sodium

sulfate was probably euglobulin. If this was true the quantities of protein precipitated at this concentration and with other methods of precipitation should agree over a wide range of concentration of protein. Comparisons were, therefore, made of the quantity of protein precipitated by 13.5 per cent of sodium sulfate at 37°C., and by saturation of the diluted serum or plasma, with carbon dioxide, and with sodium chloride at room termperature. For the carbon dioxide precipitations 5 or 0.5 cc. of serum or plasma were pipetted into a cylinder and 50 or 15 cc. respectively of distilled water added after which carbon dioxide was passed through the solution slowly for from ½ hour to 2 hours

TABLE III.

Results obtained by precipitation of different sera with (*a*) 13.5 per cent sodium sulfate, (*b*) saturated sodium chloride, and (*c*) saturation with carbon dioxide. Results are expressed as grams of nitrogen per 100 cc. of serum and represent the amount of nitrogen remaining in solution after precipitation.

Total nitrogen.	Sodium sulfate.	Sodium chloride.	Carbon dioxide.
0.658	0.595	0.621	0.573
0.665	0.665	0.661	0.578
0.693	0.600	0.569	0.621
0.695	0.613	0.615	0.652
0.696	0.630	0.615	0.652
0.731	0.595	0.661	0.600
0.863	0.718	0.792	0.735
0.901	0.665	0.718	0.569
1.046	0.915	0.929	0.915
1.255	1.000	0.997	0.957

according to the procedure of Robertson (11). The solution was then filtered and aliquot portions of the filtrate were taken for analysis. For saturated sodium chloride similar quantities of serum or plasma were taken and 50 or 15 cc. of saturated sodium chloride added after which solid sodium chloride was added in excess. These solutions were permitted to stand for 12 hours with occasional shaking. Table III contains comparative results with the three methods.

The data cover a considerable range of protein concentrations and include some sera which contain practically no euglobulin. The average difference between the results obtained with sodium

sulfate and with saturated sodium chloride is a precipitation of 0.027 gm. less of protein nitrogen per 100 cc. of blood with sodium chloride than with sodium sulfate. With relation to carbon dioxide, there was a precipitation of 0.021 gm. more protein nitrogen per 100 cc. of blood by carbon dioxide than with sodium sulfate. The average difference between results is not much beyond the experimental error but the general trend of the results is characteristic of the procedure. Similar results have been obtained in a larger number of cases with sodium chloride and sodium sulfate which give an average difference, 0.018 gm. of protein nitrogen, which is approximately the same as the one given above. Comparisons of the quantity of protein precipitated at 12.5, 13.5, and 14.5 per cent of sodium sulfate have shown repeatedly that the amount of protein precipitated at 13.5 and 14.5 per cent of sodium sulfate is essentially the same and is always more than that precipitated by 12.5 per cent of sodium sulfate. The selection of 13.5 per cent of sodium sulfate at 37°C., we believe, as nearly represents the euglobulin fraction as can be determined by such quantitative procedures.

There is an advantage in using sodium sulfate instead of carbon dioxide or sodium chloride in that very constant conditions can be maintained. With carbon dioxide losses by evaporation and frothing are to be contended with, while with sodium chloride there is always the possibility that saturation is not complete.

The Presence of Pseudoglobulin, Precipitated at 17.4 Per Cent of Sodium Sulfate, in Blood.—The presence of two pseudoglobulins in blood has often been discussed. Porges and Spiro have presented evidence in favor of the occurrence of two pseudoglobulins as the result of their precipitations from dilute serum with magnesium sulfate, sodium sulfate, ammonium sulfate, and other salts. Haslam (12) has definitely concluded that there are not more than two serum globulins in blood serum, euglobulin and pseudoglobulin. His statement is based upon results obtained after repeated precipitation of globulins with ammonium sulfate. The problem was, therefore, in such a state that it did not seem that our somewhat indefinite, but still suggestive, critical zone at about 16.4 to 17.4 per cent of sodium sulfate was significant. We are not now assured of the significance of the precipitation at 17.4 per cent of sodium sulfate. Additional evidence has been

obtained, however, which points very strongly toward the presence of a protein or protein complex whose precipitation is complete at approximately 17.4 per cent of sodium sulfate.

The evidence rests upon two facts, in addition to that already in the literature, (*a*) the indication of a critical zone at 16.4 to 17.4 per cent of sodium sulfate, already referred to, and (*b*) the *absence of protein which is precipitated at 16.4 to 17.4 per cent of sodium sulfate* in certain bloods. A precipitate first occurs at 18.4 per cent of sodium sulfate. Under suitable conditions the blood of the same animals will contain, within a few hours, large quantities of protein precipitable at 13.5 per cent of sodium sulfate, or by carbon dioxide, or saturated sodium chloride, and at 17.4 per cent of sodium sulfate. Other work associated with this and confirming this observation will be presented in due time.

The presence of a protein in this case rests upon the acceptance of the definition of a euglobulin as one which is precipitated by acidification with carbon dioxide in dilute solution. That the amount of protein precipitated by carbon dioxide from blood is practically the same as that obtained under two other conditions has just been discussed and points to the separation of at least a mixture of fairly definite composition. This being so the failure to obtain a precipitate until at least 18.4 per cent of sodium sulfate has been added to blood serum under some conditions and the presence of a precipitate under others is an indication of the existence of a protein or protein complex precipitable between the limits of 13.5 and 17.4 per cent of sodium sulfate.

The quantity of protein precipitated between 13.5 and 17.4 per cent of sodium sulfate in one series of experiments is roughly equal to that precipitated at 13.5 per cent. There are bloods in which the quantity of protein precipitated between these limits is greater than at 13.5 per cent of sodium sulfate. Judging from the volume of precipitate the greatest proportion of the precipitation may occur between 16.4 and 17.4 per cent of sodium sulfate. This fact made us hesitate between 16.4 and 17.4 per cent of sodium sulfate as the percentage which would represent the approximate completion of the precipitation of pseudoglobulin I. On the other hand, there are cases in which no precipitate occurs at 17.4 per cent of sodium sulfate. When the crit-

ical zone is most marked at the range of concentrations under consideration, 17.4 per cent of sodium sulfate is always one of the two concentrations involved and we have, therefore, adopted this concentration as the point which we consider as best representing the quantity of euglobulin plus pseudoglobulin I present in blood. In case plasma is being studied the value at 17.4 per cent will represent fibrinogen, euglobulin, and pseudoglobulin I.

DISCUSSION.

Data have been presented which indicate that as increasing quantities of sodium sulfate are added to diluted serum or plasma or serum at 37°C. the amount of protein thrown out of solution increases. There are at least three points in such a series at which an increase of 1 per cent of sodium sulfate does not produce as large an increase in precipitation as will be caused by the concentration of salt preceding or succeeding these concentrations. These points, or critical zones, are at 13.5 to 14.5, 16.4 to 17.4, and 21 to 22 per cent of sodium sulfate. This is particularly true of the concentrations 13.5 to 14.5 and 21 to 22 per cent of sodium sulfate. At 16.4 to 17.4 per cent of sodium sulfate it is not always possible to demonstrate a critical zone.

Basing our conclusions on the quantity of protein precipitated as determined by analysis of the filtrate from such precipitations[4] two of these critical zones agree very closely with other methods for determining proteins. Results at the zone at 13.5 to 14.5 per cent of sodium sulfate agree closely with those obtained with saturated sodium chloride and carbon dioxide. At 21 to 22 per cent of sodium sulfate the results are approximately those obtained with magnesium sulfate and ammonium sulfate both of which have long been accepted as a means of completely

[4] In using the procedures described it is assumed that the aliquot taken from the filtrate after precipitation contains a true proportionate amount of the unprecipitated protein and that there has not been any adsorption of the unprecipitated protein by the precipitated protein nor by the filter paper. The results of Spiro (13) indicate that precipitation is probably not complete and that there is a small amount of protein remaining unprecipitated. We have found that when diluted serum is filtered that there is a small loss of nitrogen in the process which may be slightly greater than the experimental error.

precipitating globulins. For the acceptance of the zone at 17.4 per cent of sodium sulfate the evidence rests in part upon the absence of protein precipitable up to that concentration in certain bloods.

Whether or not results obtained by precipitation of proteins from a mixture of proteins with salts represent separations of pure proteins is an open question. The considerable mass of literature on this subject is in favor of the opinion that the protein thrown down is a mixture of proteins; (*a*) present as compounds, (*b*) due to the adsorption of other proteins by the precipitated protein, or (*c*) because the precipitation limits overlap. One fact stands out in our experiments, however, which is applicable to any concentration of salt; under constant conditions of temperature and concentration of salt a constant amount of protein is precipitated. Robertson (11) in developing his refractometric procedure for the determination of globulins substantiated his use of ammonium sulfate for the precipitation of total globulins upon the constancy of the results obtained with definite concentrations of salt. From our subsequent work with a method developed from our findings presented here we agree with Robertson in referring to precipitation with ammonium sulfate, that "if the proportion of this substance is different in the serum of different individuals or species, we may be fairly confident, therefore, that the quantitative relations of the globulin and albumin," or intermediate globulin, "groups are different in these animals."

Our work has covered a number of species of mammals having blood of varying protein content and the limits given have held in every case. On the other hand, in carrying out a problem involving the determination of serum or plasma proteins we would strongly advise the use of two succeeding concentrations of salt at the critical zone until it had been determined that the critical zone was present at the concentrations of salt used in that particular case.

SUMMARY.

1. Sodium sulfate solutions at 37°C. may be used to precipitate the proteins of blood into fractions corresponding to those usually separated by carbon dioxide or saturated sodium chloride, euglobulin, and magnesium sulfate or ammonium sulfate, total globulin.

2. Critical zones in the curve representing the precipitation of protein with increasing salt concentration have been located at 13.5 to 14.5, 17.4, and 21 to 22 per cent of anhydrous sodium sulfate at 37°C. For the purpose of estimating the quantity of protein present at these zones, 13.5, 17.4, and 21.5 per cent of sodium sulfate is recommended.

3. Evidence has been presented which tends to substantiate the presence of two globulins in blood serum in addition to euglobulin; pseudoglobulin I and pseudoglobulin II whose precipitations are complete at approximately 17.4 and 21 to 22 per cent of sodium sulfate respectively.

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THE DETERMINATION OF PROTEINS IN BLOOD—A MICRO METHOD.

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The use of sodium sulfate instead of ammonium sulfate in the precipitation of globulins makes it possible to determine the proteins of plasma or serum in small quantities of blood. The basis for the selection of sodium sulfate has been discussed in the preceding paper (1). Robertson (2) described a procedure for the determination of blood proteins in small quantities of blood with the aid of the refractometer. Cullen and Slyke (3) have proposed a procedure which gives consistent results and which does not require any special apparatus beyond that to be found in any laboratory. Their method, however, required 5 cc. of plasma for each constituent of the blood determined. The determinations described below can be performed with the usual laboratory apparatus and require but 0.5 cc. of plasma or serum for each determination.

The procedures involve the precipitation of fibrinogen with calcium chloride, the globulins with definite concentrations of sodium sulfate at 37°C., and non-protein nitrogen with trichloroacetic acid. In the case of fibrinogen and non-protein nitrogen the technique of Cullen and Van Slyke is followed. The globulins are precipitated by adding a concentration of sodium sulfate which is greater than the required percentage by the amount of sodium sulfate necessary to produce the desired percentage when added to the blood sample. The solutions are prepared by dissolving the required quantity of sodium sulfate in a little less than the final volume, which requires heat for the higher percentages, and then diluting to volume at 37°C. All precipitations and filtrations with sodium sulfate are carried out in the

incubator or hot room. The following concentrations of sodium sulfate are needed: 14, 18, and 22.2 per cent. When 15 cc. portions of these solutions are added to 0.5 cc. of blood the final concentrations are approximately 13.5, 17.4, and 21.5 per cent of sodium sulfate respectively. At 13.5 per cent of sodium sulfate euglobulin is precipitated, at 17.4 per cent euglobulin and pseudoglobulin I are precipitated, and at 21.5 per cent all globulins are thrown out of solution. In case blood plasma is used fibrinogen is present in each case and the nitrogen representing this protein must be deducted. Suitable blanks must be made for each determination.

The volume of solution used, 15.5 cc. (15 cc. of salt solution plus 0.5 cc. of blood), permits duplicate determinations to be made on each precipitation. This does not insure against errors in precipitation, but it has been our experience that simultaneous duplicate precipitations almost invariably agree. When it is desired to make duplicate precipitations it is advised that 13.5 and 14.5 per cent, 16.4 and 17.4 per cent, and 21 and 22 per cent of sodium sulfate, final concentrations, be used. With these concentrations the values obtained with each pair should agree within experimental error, except perhaps in the case of 16.4 and 17.4 per cent of sodium sulfate.

Precipitations are made in test-tubes or 50 cc. centrifuge tubes and then closed with a rubber stopper. The filtrations are conducted in the incubator using a dry 9 cm. filter paper. It is desirable to wet the filter paper with a small amount of the solution to be filtered before pouring on the bulk of the solution containing the precipitate. The funnels are covered with watch-glasses. 1 inch test-tubes held in test-tube racks are convenient for filtration. With these tubes and the cover-glasses a reasonably tight filtration system is obtained.

For measuring, the accurately calibrated Ostwald pipettes and the 15 cc. graduated pipettes introduced by Folin are used.

The nitrogen determinations are conducted in large Pyrex test-tubes in general according to the original micro procedure of Folin and Farmer (4), and the distillations are carried out, according to the procedure of Folin and Wu (5) in their system of blood analysis, without cooling the distillate. In distilling, a Pyrex connecting tube is used which carries a distilling head and

has an enlargement on the tube which dips into the acid to guard against mechanical transfer of alkali and back suction. For titrations we use standard hydrochloric acid and sodium hydroxide which are approximately 0.05 and 0.025 N respectively. Our burettes deliver 25 cc. and are graduated to 0.05 cc. Methyl red is used as an indicator.

The determinations are as follows: Plasma is collected so that it contains 0.5 per cent of potassium oxalate. Both plasma and serum are centrifuged until clear.

Total Nitrogen.—0.5 cc. of plasma or serum is placed in a large Pyrex test-tube and the 2 cc. of concentrated sulfuric acid, 1 drop of 5 per cent copper sulfate, and a quartz pebble are added; the solution is digested over a free flame until clear, and then 7 to 10 minutes longer. Cool 3 to 5 minutes, add 25 to 30 cc. of ammonia-free distilled water, a small amount of talcum powder or powdered pumice stone, and concentrated sodium hydroxide solution sufficient to neutralize the acid, and distill into standard acid.

In place of using two 0.5 cc. portions of blood 15 cc. of 0.8 per cent sodium chloride solution may be added to one portion of 0.5 cc. and two 5 cc. portions of the diluted plasma taken for analysis.

Fibrinogen.—0.5 cc. of plasma is measured into a tube, 14 cc. of 0.8 per cent sodium chloride solution at room temperature are added, then 1 cc. of 2.5 per cent calcium chloride, a small crystal of thymol, and the tube is stoppered. The tube and contents are allowed to stand until the fibrin is formed and then filtered on a dry filter. Two 5 cc. portions of the filtrate are taken for analysis.

Euglobulin.—0.5 cc. of plasma or serum is measured into a tube, 15 cc. of 14 per cent anhydrous sodium sulfate at 37°C. and a little thymol are added, and the tube is stoppered, shaken, and allowed to stand for at least 3 hours, or until the precipitate has settled. The solution is then filtered through a dry filter and two 5 cc. portions are taken for analysis. The results represent euglobulin in the case of serum and fibrinogen plus euglobulin in the case of plasma.

Euglobulin Plus Pseudoglobulin I.—The procedure is the same as for euglobulin except that 18 per cent sodium sulfate is used.

Total Globulins.—The procedure is the same as in euglobulin except that 22.2 per cent of sodium sulfate is used.

Non-Protein Nitrogen.—0.5 cc. of plasma or serum is measured into a tube and 15 cc. of 5 per cent trichloroacetic acid at room temperature are added. The remainder of the procedure is the same as in euglobulin.

The calculations of nitrogen are those ordinarily associated with Kjeldahl determinations. The volume of solution from which the aliquot portions for analysis are taken is 15.5 cc. We have expressed our results in terms of grams of nitrogen in 100 cc. of blood. As the result of the analytical procedures the following results can be calculated for serum:

Total nitrogen.

Euglobulin nitrogen = Total nitrogen − nitrogen in filtrate from 13.5 per cent sodium sulfate precipitation.

Pseudoglobulin I nitrogen = Nitrogen in filtrate from 13.5 per cent sodium sulfate precipitation − nitrogen from 17.4 per cent sodium sulfate precipitation.

Pseudoglobulin II nitrogen = Nitrogen in filtrate from 17.4 per cent sodium sulfate precipitation − nitrogen in filtrate from 21.5 per cent sodium sulfate precipitation.

Total globulin nitrogen = Total nitrogen − nitrogen in filtrate from 21.5 per cent sodium sulfate precipitation.

Albumin nitrogen = Nitrogen in filtrate from 21.5 per cent precipitation − non-protein nitrogen.

Non-protein nitrogen = Nitrogen in filtrate from trichloroacetic acid precipitation.

For plasma the euglobulin is estimated by subtracting the filtrate nitrogen from the nitrogen in the filtrate after the precipitation of fibrinogen.

Table I contains some comparative data on the Cullen-Van Slyke procedure and the micro method. Determinations using sodium sulfate and magnesium sulfate in the Cullen-Van Slyke method are included. Determinations for pseudoglobulin I are not included in the table since they were not made at the time the plasma was analyzed. The results are expressed in terms of the nitrogen remaining in the filtrate after precipitation without calculating the various fractions.

TABLE I.

Comparative results obtained with the method of Cullen and Van Slyke "macro" and the micro method. Results are expressed as grams of nitrogen per 100 cc. of blood remaining in the filtrate after precipitation.

	Cow.		Pig.		Goat.		Horse.	
	Macro.	Micro.	Macro.	Micro.	Macro.	Micro.	Macro.	Micro.
Total nitrogen......		1.67	1.05	1.05		1.10		1.08
$CaCl_2$...............	1.31	1.39	0.91	0.92	0.98	0.97	1.03	0.97
Saturated NaCl.....	1.01	1.00	0.80	0.81	0.84	0.83	0.90	0.89
Sodium sulfate.								
13.5 per cent....		1.01		0.74		0.84		0.88
20.9 " "	0.31		0.41		0.39		0.43	
21.3 " "		0.29		0.39		0.40		0.44
22.5 " "		0.27		0.40		0.38		0.43
23.2 " "		0.27		0.31		0.37		0.41
Trichloroacetic acid.	0.06	0.07	0.03	0.02	0.05	0.07	0.04	0.06
Ammonium sulfate..			0.53		0.44		0.48	
Magnesium sulfate..					0.32		0.39	

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4. Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.
5. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

AN EFFECT OF THE INGESTION OF COLOSTRUM UPON THE COMPOSITION OF THE BLOOD OF NEW-BORN CALVES.

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(Received for publication, September 22, 1921.)

An interesting relation between the ingestion of colostrum and the composition of the blood of new-born calves was observed in the course of a study in progress in this department. It was found that the blood serum of a new-born calf before it has nursed does not contain proteins precipitable by 17.4 per cent anhydrous sodium sulfate, or at least but a trace of precipitation occurs at this concentration. Precipitates do not occur with concentrations less than 17.4 per cent of sodium sulfate, with saturated sodium chloride, nor with carbon dioxide. If we accept the usual means of separating the proteins of blood the failure to obtain a precipitate under the above conditions amounts to saying that the blood of a new-born calf *does not contain euglobulin nor pseudoglobulin I*. After the calf has received colostrum the blood serum contains relatively large amounts of protein precipitable by carbon dioxide, saturated sodium chloride, and 13.5 per cent sodium sulfate—euglobulin—and also at 17.5 per cent of sodium sulfate—pseudoglobulin I.[1] On the other hand, if the calf is given ordinary whole milk from a cow well along in lactation, or receives milk from a cow which has not been "dried off" before parturition, *i.e.* has been milked up to or close to the time of parturition, conditions in which very little globulin is ingested, the quantity of euglobulin or pseudoglobulin in the blood is negligible. The subsequent ingestion of colostrum after

[1] These proteins may be absent from the blood of older calves. The conditions under which this occurs have not been, as yet, determined. So far as we have examined the blood of adult animals, the serum always contains euglobulin and pseudoglobulin I.

TABLE I.

Data Showing the Relation between the Ingestion of Colostrum and the Composition of the Blood of New-Born Calves. Results are Expressed as Gm. of Nitrogen in 100 Cc. of Blood.

Remarks.	Total nitrogen.	Euglobulin.	Pseudo-globulin I.	Pseudo-globulin II.	Total globulin.	Albumin.	Non-protein nitrogen.
Calf A.							
5 hrs. after birth. No colostrum.	0.596	0.023	0.004	0.140	0.167	0.376	0.053
6 hrs. after receiving colostrum. Age 11 hrs.	0.838	0.173	0.235	0.087	0.505	0.280	0.053
1 day old	1.028	0.337	0.217	0.131	0.765	0.210	0.053
2 days "	0.962	0.271	0.310	0.075	0.656	0.253	0.053
Calf B.							
5 hrs. after birth. No colostrum.	0.605	0.000	0.009	0.140	0.141	0.390	0.074
16 hrs. after receiving whole milk. Age 21 hrs.	0.633	0.000	0.040	0.135	0.173	0.386	0.074
5 hrs. after receiving colostrum from mother. Age 26 hrs.	0.632	0.000	0.074	0.140	0.216	0.363	0.053
17 hrs. after receiving colostrum. Age 43 hrs.	0.668	0.032	0.084	0.101	0.222	0.393	0.053
4 days old	0.687	0.048	0.144	0.110	0.312	0.332	0.053
Calf C.							
At birth	0.595	0.000	0.096*	0.101	0.197	0.350	0.048
6 hrs. after birth. No milk	0.625	0.000	0.097*	0.083	0.157	0.415	0.053
17 hrs. after nursing. Mother not "dried off"	0.647	0.039		0.192	0.231	0.363	0.053
24 hrs. after nursing mother. Age 47 hrs.	0.668	0.003	0.168	0.022	0.183	0.332	0.053
2 days old	0.643	0.032	0.153	0.035	0.223	0.367	0.053
3 " "	0.611	0.051	0.149	0.035	0.235	0.323	0.053

* In these cases there was practically no protein precipitated at 16.4 per cent of sodium sulfate. The results given are based on data obtained at 17.4 per cent of sodium sulfate.

a period, so far observed, of 21 hours results in the appearance of the globulins in the blood. The above facts relate to blood serum. Fibrinogen is present in the blood of a new-born calf and is precipitated under all of the conditions mentioned above.

In the study of the changes which take place in the blood of young calves the recently published method[2] was used, which involves the use of sodium sulfate at 37°C. The data of Table I serve to illustrate the changes which take place in the composition of the blood of the calf during the early days of its life.[3]

Calf A received colostrum within 5 hours after birth. Calf B was fed whole milk 5 hours after birth and then its mother's colostrum which had been held in the refrigerator for 16 hours. The mother of Calf C was milked up to the time of parturition.

The absence of euglobulin and pseudoglobulin I from the blood of new-born calves and its sudden appearance after ingesting colostrum brings the relation of colostrum to the new-born into a new light. It would appear that the calf receives its first supply of these globulins from the colostrum and that if they are not acquired from the colostrum it probably takes some time to form them. We are now studying the conditions under which these globulins are produced.

It has repeatedly been shown that colostrum is rich in globulins and that later the quantity of globulin in milk is practically negligible; the presence of globulin has, in fact, often been questioned. The function of colostrum has not been well understood; the most common explanation is that it acts as a purgative. It has been observed that an animal can live and grow without colostrum. Hohlfeld[4] found that dogs, goats, and guinea pigs could be raised without receiving colostrum but that better

[2] Howe, P. E., *J. Biol. Chem.*, 1921, xlix, 109.

[3] There is some difference of opinion with regard to the significance of results obtained by precipitation of proteins from a mixture of proteins, such as blood serum, due to the overlapping of the precipitation limits or the adsorption of unprecipitated protein by the precipitated protein. The data in Table I indicate that for comparative studies the results have some significance. The wide range in variation in the amount of globulins does not appear to have the marked effect upon the various fractions estimated which one would expect if the interfering factors played an important rôle in the processes involved.

[4] Hohlfeld, M., *Arch. Kinderheilk.*, 1907, xlvi, 161.

growth in early life occurred when colostrum was taken. He ascribed the value of colostrum to its higher nutritive value, which enabled the young with a small stomach capacity to obtain sufficient nourishment in a relatively small volume. We have observed the effect of the absence of colostrum upon the feces content of young calves[5] and found that the appearance of an increased fat content which occurs normally in about 3 days was delayed but that it appeared subsequently.

It is a pleasure to record our thanks to Dr. R. B. Little for the opportunity of analyzing blood serum collected by him in the course of his experiments.

[5] Howe, P. E., *Am. J. Dis. Child.*, 1921, xxi, 57.

VITAMINE CONTENT OF RICE BY THE YEAST METHOD.

ORGANIC NITROGEN AS A POSSIBLE FACTOR IN STIMULATION OF YEAST.

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(Received for publication, August 24, 1921.)

Although the etiological rôle of rice in connection with beri-beri was among the first considerations which opened up the entire subject of vitamines, recent investigations have paid but little attention to rice as a source of water-soluble B. It was understood that if the diet was not sufficiently supplied with other sources of water-soluble B, the rice must retain enough of the pericarp to supply this deficiency, but quantitative expression of the water-soluble B so supplied was wanting. Recently the writer had occasion to estimate the water-soluble B content of a particular lot of rice which was supplied to troops so situated as to make this source of the vitamine of importance. The estimation was attempted by the yeast cell method then being published by several investigators. The results are submitted as an example of practical application of the method and as a suggestion of one possible factor upon which the stimulation of yeast growth depends.

The technique of Fulmer, Nelson, and Sherwood[1] was used for estimating the growth of the yeast, the "count," as mentioned hereafter, being the same as theirs; number of cells seen in 16 small squares of the blood counting chamber. An extract of Fleischmann's yeast in 0.1 per cent acetic acid was used as a standard source of water-soluble B. For purposes of comparison, extracts in 0.1 per cent acetic acid of samples of rice of the same growing but in varying stages of milling and polishing were used.

[1] Fulmer, E. I., Nelson, V. E., and Sherwood, F. F., *J. Am. Chem. Soc.*, 1921, xliii, 186.

The rice under investigation was likewise used in extract in 0.1 per cent acetic acid.

The results of the cultures after additions of these extracts were similar to the results of similar experiments by others and need not be given in detail. In general the growth of the yeast was proportional to the amount of extract added, and the growth was not only greater with increasing amounts of extract but, in the case of yeast and of rice supposedly rich in water-soluble B—unhusked and brown rice—the rate of increase of growth per unit weight of extract added was greater.

However, when these stimulating extracts were so treated with alkali as to destroy any water-soluble B they might contain, results were obtained which threw grave doubts on the growth of yeast being a measure of the water-soluble B content of the culture medium. After addition of sodium hydroxide to the extract to a concentration of 10 per cent, boiling the extract almost to dryness, neutralizing with concentrated hydrochloric acid, and making up to the original volume with 0.1 per cent acetic acid, such an extract produced as great a growth as before alkali treatment, and in some cases even greater growth. This was regarded as throwing sufficient suspicion on the method as a quantitative measure of water-soluble B to cause it to be abandoned for the purpose of the original inquiry. An opinion on the suitability of the rice was rendered based on the amount of pericarp present as evidenced by the iodine test and the percentage of fiber, fat, and phosphorus present.[2,3]

In seeking an explanation of the decided stimulation of yeast growth by these extracts, both before and after treatment with alkali, it occurred to the writer that the addition of nitrogen in organic form in the extract to the culture medium in which the nitrogen is present solely as inorganic nitrogen might be a factor in this stimulation. It will be remembered that it is a basic principle of the yeast method that the nitrogen of the culture medium be derived solely from inorganic sources.

In investigating this point, the total nitrogen of several of the extracts was determined, using the Folin micro-Kjeldahl technique

[2] Vedder, E. B., Beriberi, New York, 1913, 87.

[3] Leach, A. E., Food inspection and analysis, New York, 3rd edition, 1913, 272, 277, 301, 346.

as used in this laboratory for determination of the non-protein nitrogen of blood. With these values and with the counts produced by these extracts in the growth experiments two ratios were derived:

$$\text{Ratio I.} \quad \frac{\text{Count for } n \text{ mg. of extract}}{\text{Nitrogen in } n \text{ mg. of extract}}$$

$$\text{Ratio II.} \quad \frac{\text{Nitrogen in } n \text{ mg. of extract}}{\text{Nitrogen as } NH_4Cl \text{ in culture flask}}$$

TABLE I.

Material extracted.		Weight of material added to flask.					Nitrogen per 100 mg.
		5 mg.	10 mg.	25 mg.	50 mg.	75 mg.	
Rice bran, Rickert.	Count.....	70	63	82	58	134	1.75
	Ratio I....	800	360	186	66	101	
	Ratio II...	0.0038	0.0077	0.019	0.0341	0.058	
Rice, fancy head, Rickert.	Count.....	29	28	24	30	42	0.56
	Ratio I....	1,040	500	171	107	100	
	Ratio II...	0.0012	0.0025	0.0062	0.0124	0.0185	
Rice, brown, Grosjean.	Count.....	11	42	40	54	60	0.85
	Ratio I....	259	520	187	126	94	
	Ratio II...	0.0019	0.0037	0.0094	0.0188	0.0278	
Rice, 1st break, Grosjean.	Count.....	24	30	20	46	83	0.55
	Ratio I....	870	545	146	167	201	
	Ratio II...	0.0012	0.0024	0.0061	0.0121	0.0181	
Rice, QMC contract.	Count.....	18	14	14	37	43	0.77
	Ratio I....	465	172	72	130	74	
	Ratio II...	0.0017	0.0034	0.0085	0.017	0.0255	
Yeast.	Count.....	27	32	71	82	112	1.69
	Ratio I....	320	200	168	97	88	
	Ratio II...	0.0037	0.0074	0.018	0.0374	0.056	

The second ratio gives the percentage of organic nitrogen to inorganic nitrogen in the culture flask. (46 cc. of culture medium in each flask.) The values of these two ratios as found for various amounts of several extracts are given in Table I.

These ratios show wide variations it is true. Still it is believed these variations fall sufficiently within the experimental variation of the method itself to suggest that the addition of organic nitrogen to the culture medium is a factor in the stimulation of yeast growth by extracts of organic substances. It will be noted that the lowest concentration of this organic nitrogen in Ratio II of Table I is 0.1 per cent and that it runs as high, in the case of the higher counts, as 5.8 per cent. It is believed that this concentration of organic nitrogen is too high to be a negligible factor in a method a basic requirement of which is that the nitrogen of the culture medium shall be entirely inorganic. The values for Ratio I for equal amounts of extract also show a suggestive constancy.

SUMMARY.

1. In an attempt at quantitative estimation of the water-soluble B content of rice, the yeast method was abandoned, the results of alkali treatment of the stimulant extracts being regarded as disproving a specific action of water-soluble B in stimulation of yeast growth.

2. Evidence is submitted that the addition of organic nitrogen to the inorganic nitrogen of the culture medium is one factor in the stimulation of yeast growth.

THE CATALYTIC EFFECT OF AMMONIA ON THE OXIDATION OF BUTYRIC ACID WITH HYDROGEN PEROXIDE.

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(Received for publication, July 18, 1921.)

The results described in this paper bring to light what appears to be a specific effect of ammonium hydroxide on the oxidation of butyric acid by hydrogen peroxide. The results are of such a nature that it appears that they aid in interpreting fatty acid oxidation in acidosis of the diabetic type.

The experiments here described grew out of a discordance between the author's own previous results[1] on the influence of varying amounts of potassium hydroxide on the oxidation of butyric acid with hydrogen peroxide and those of Dakin[2] on the oxidation of ammonium butyrate with hydrogen peroxide. In my own experiments it was found that the acetone yield increased as the amount of potassium hydroxide present diminished, but was never large. Dakin did not vary the amount of ammonium hydroxide used, but obtained many times as much acetone as was obtained under similar conditions with potassium hydroxide in my own experiments. Under these conditions, it seemed advisable to run simultaneous comparative experiments on the influence of ammonium and potassium or sodium hydroxides, particularly since specific effects of the salts of these bases on biological processes have become better known since the time of Dakin's publications.[3] Both Dakin's and my own previous experiments were fully confirmed. The new experiments devel-

[1] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 83.

[2] Dakin, H. D., *J. Biol. Chem.*, 1908, iv, 77.

[3] *Cf.* Amberg, S., and Helmholz, H. F., *J. Pharm. and Exp. Therap.*, 1918, xii, 19 for data and many references.

oped give additional interest to the careful experimental studies of Dakin on the oxidation of butyric acid. On the basis of the results described here, it seems not impossible that he was studying the actual mechanism of fatty acid oxidation in the diabetic type of acidosis, or at least a mechanism generically related to it.

EXPERIMENTAL.

Methods of Analysis.—When the large number of possible products of oxidation of butyric acid is considered, it is obvious that a complete quantitative analysis by the crude methods available, even for such of these compounds as are chemically known, is still out of the question. The problem, therefore, was to make such analytical determinations as would give an unmistakable indication of the state and trend of the oxidation. Suitable simple ways of determining unchanged butyric acid, acetone, and carbon dioxide were adopted and were found to give the needed information.

A portion of the oxidation solution (generally 100 or 110 cc.), with 50 cc. of water and 2 gm. of powdered manganese dioxide, was placed in a long necked distilling flask attached to a long condenser. It is necessary to use some catalyst that decomposes peroxide rapidly so that the results may be comparable, but especially to prevent the volatilization of the peroxide, which interferes with the acetone titration. A Wolff bottle connected by means of an adapter constituted the receiver. The other tubulature of the Wolff bottle was connected to two low wide mouthed wash bottles. Two wash bottles, containing sodium hydroxide and barium hydroxide, respectively, were attached to the distilling flask. During distillation a suction pump attached at the lower end circulated CO_2-free air into the apparatus and transported the CO_2 evolved on warming the mixture in the flask into the barium hydroxide solution. When only about 50 cc. remained in the distilling flask distillation was interrupted. The flask contents were filtered to remove the manganese dioxide, the filter was washed, about 50 cc. of water were added, the solution was strongly acidified with phosphoric acid, and distillation was resumed. In this way small additional amounts of CO_2 were obtained and also unchanged butyric acid as well as other volatile acids were driven over.

The CO_2 formed was determined by filtering off and weighing the $BaCO_3$ precipitated. The acetone was determined by using an aliquot portion of the distillate for the Lieben iodoform titration and the iodine consumed was calculated as acetone. This does not give the correct amount of acetone, since other iodoform-producing compounds are also present. The results, however, are satisfactory for comparative purposes.

The rapid determination of unchanged butyric acid in the distillate, even approximately, gave more difficulty until the rough method given below was adopted. The other possible volatile acids that would most likely interfere are propionic, acetic, and formic acids. Fortunately, all these acids in dilute aqueous solutions, such as were used here, have a typical form of volatility curve as was shown in another connection in a previous paper.[4] On the basis of these curves and in the absence of a better method, the attempt was made to utilize this characteristic behavior on distillation to identify the main volatile acid products and to determine them approximately at the same time.[5]

In order to determine whether this volatility curve can be used for the determination of unchanged butyric acid three distillations with known amounts of butyric acid were carried out. The amount of butyric acid given in the table was made up to 225 cc. with distilled water. Four fractions of 50 cc. were distilled off and titrated with 0.1 N NaOH.

	A	B	C
Butyric acid used, *gm*.	0.25	0.25	0.125
Fraction 1, *cc*.	10.50	10.92	5.38
" 2, *cc*.	8.04	8.08	4.08
" 3, *cc*.	5.60	5.52	2.75
" 4, *cc*.	3.30	3.20	1.64

More concordant results than these were repeatedly obtained without any special care. It will be noted that if the amount of butyric acid had been unknown in any case it could have been

[4] Witzemann, E. J., *J. Am. Chem. Soc.*, 1919, xli, 1948.

[5] Duclaux, M., *Ann. chim. phys.*, 1874, ii, 289; *Ann. Inst. Pasteur*, 1895, ix, 265. Upsom, F. W., Plum, H. M., and Schott, J. E., *J. Am. Chem. Soc.*, 1917, xxxix, 731.

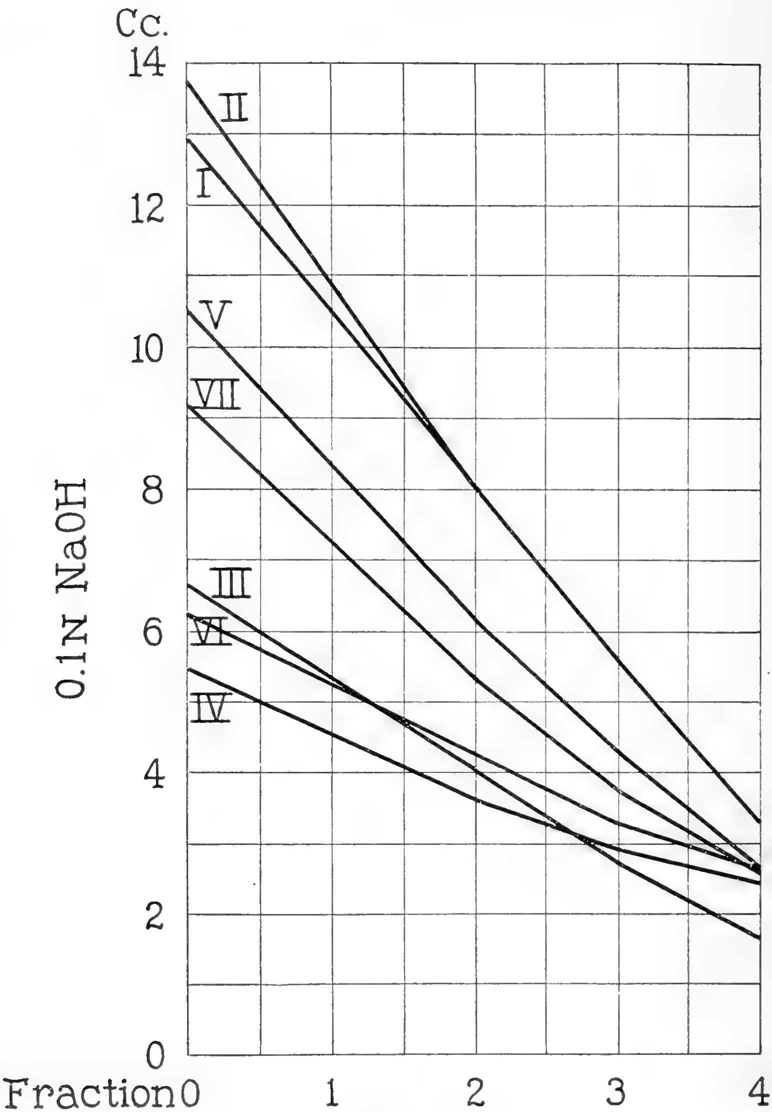

FIG. 1. Volatility curves.

determined approximately from the titration values in the other columns.

The results are given graphically in Curves I, II, and III in Fig. 1 for comparison with the curves of unknowns.

The method proved satisfactory for the purposes of this paper and was valued because the determination could be made in 15 or 20 minutes.

The data on the butyric acid recovered in such experiments are of especial importance for two reasons. (1) They enable one to determine approximately how much oxidation of all kinds has taken place. (2) Acetone is subject to oxidation in alkaline solutions with hydrogen peroxide[6] and the acetone found would not necessarily be a satisfactory index of the amount of oxidation that occurred. Owing, moreover, to the fact that enolization precedes oxidation in the case of acetone[7] the velocity of this oxidation of acetone would probably be greater when potassium is used than when ammonium hydroxide is used and so lower yields of acetone would be expected with the former. The data as determined prevent any such misunderstanding of the results.

In the preliminary qualitative experiments given below only the first distillation was carried out and no effort was made to determine the unchanged butyric acid and the carbon dioxide formed. Later the methods described above were developed and used.

It is well known that ammonium reacts in various ways with hypochlorous acid and alkali hypochlorites, hypobromites, and hypoiodites in such a way that oxidation of ammonia results. This process is apparently perceptible on adding 0.1 N iodine solution to a solution strongly alkaline with sodium hydroxide containing small amounts of ammonia. Test experiments showed that although small, the error due to such reactions in the titration of acetone was increasingly significant as the amount of acetone present diminished. This error can be avoided by adding excess phosphoric acid to the distillate containing ammonia and distilling again. Moreover, if the excess is sufficient the butyric acid is quantitatively recovered. In all experiments reported in this

[6] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 89.

[7] Witzemann, E. J., *J. Am. Chem. Soc.*, 1917, xxxix, 2657.

paper 10 cc. of syrupy phosphoric acid were used in this extra distillation for the removal of ammonia.

For the sake of convenience and accuracy the butyric acid used in all experiments was converted into a 5 per cent aqueous solution so that 5 cc. corresponded to 0.25 gm. of the acid. Similar solutions of ammonium, potassium, and sodium hydroxides were prepared of which 5 cc. corresponded to 0.25 gm. of butyric acid.

Influence of Ammonium Hydroxide.—In the following experiments with mixtures of butyric acid and potassium butyrate no marked difference in the yield of acetone was observed. Aqueous solutions, 100 cc. in volume, containing 50 cc. of 3 per cent hydrogen peroxide and butyric acid as given below were allowed to stand 42 to 45 hours at room temperature and analyzed. (*a*) Contained 0.25 gm. of butyric acid as the K salt. (*b*) Contained 0.20 gm. of butyric acid as the K salt and 0.05 gm. of free butyric acid. (*c*) Contained 0.15 gm. of butyric acid as the K salt and 0.10 gm. of free butyric acid. (*d*) Contained 0.25 gm. of free butyric acid.

In all cases the iodine consumed by the distillate corresponds to about 0.001 to 0.002 gm. of acetone. In other words, the acetone formed is practically imperceptible in all cases.

A similar series of experiments was done with ammonium butyrate. The ammonium butyrate was prepared by neutralizing a butyric acid solution of known strength with ammonium hydroxide using some excess of ammonia. 100 cc. of solution contained 50 cc. of 3 per cent hydrogen peroxide and

(1) With 0.25 gm. of butyric acid as NH_4 salt	yielded	0.0133	gm.	of	acetone.
(2) With 0.20 gm. of butyric acid as NH_4 salt and 0.05 gm. of free butyric acid	"	0.0123	"	"	"
(3) With 0.15 gm. of butyric acid as NH_4 salt and 0.10 gm. of free butyric acid	"	0.0053	"	"	"
(4) With 0.25 gm. of free butyric acid	"	0.0041	"	"	"

These results show that the presence of the ammonium radical causes a marked increase in the yield of acetone. This result is in sharp contrast to what was regularly observed with potassium

butyrate as compared with butyric acid, as indicated by results such as those given above.

Another series with 0.25 gm. of butyric acid and 50 cc. of 3 per cent hydrogen peroxide in 100 cc. of solution and containing ammonium hydroxide as follows:

(5)	With 50 cc. of NH_4OH solution yielded	0.0096 gm. of acetone.				
	(5 cc. ≡ 0.25 gm. of butyric acid.)					
(6)	With 25 cc. of NH_4OH solution yielded	0.0076	"	"	"	
(7)	" 10 " " NH_4OH " "	0.0069	"	"	"	
		0.0075	"	"	"	
		0.0093	"	"	"	
(8)	" 7.5 " " NH_4OH " "	0.0075	"	"	"	
(9)	" 6.25 " " NH_4OH " "	0.0030	"	"	"	
(10)	" 5.00 " " NH_4OH " "	0.0021	"	"	"	
		0.0017	"	"	"	
(11)	" 2.5 " " NH_4OH " "	0.00138	"	"	"	
(12)	" 0.0 " " NH_4OH " "	0.0016	"	"	"	
		0.00153	"	"	"	

These experiments constitute parts of three different series in which all of the reagents used and the solutions for titration were different. Although there is apparently considerable variation, the results conform with the preceding series. The results with (5), (6), and (7) suggest that apparently the maximum effect of the ammonium hydroxide is being exerted since the increase in the acetone is nowhere nearly proportional to the increase in ammonium hydroxide.

Repetition of (5), (6), and (10), brought out the fact that after 67 hours at room temperature, all peroxide had disappeared from (5), all but a trace from (6), while (10) still contained much peroxide. On distilling the mixtures in the presence of 2 gm. of manganese dioxide, the following results were obtained:

(5)	0.01887 gm. of acetone	(11.5 per cent yield).
(6)	0.0258 " " "	(15.7 " " ").
(10)	0.00523 " " "	(3.2 " " ").

By means of other experiments which will not be described here, it was found that ammonium hydroxide catalyzes the rapid decomposition of hydrogen peroxide while potassium hydroxide does not do so. The smaller amount of oxidation in (5) as compared with (6) is due to the fact that more oxygen was lost into

the air in the latter case. This simply means that the oxidation reaction is not increased at the same rate by additional ammonium hydroxide as the decomposition reaction when the concentration of the other components remains the same.

The evolution of oxygen is visible in both (5) and (6), but not in (10).

The difference in the effect of potassium hydroxide as compared with ammonium hydroxide is strikingly brought out by the following experiments in which 0.25 gm. of butyric acid and 50 cc. of 3 per cent hydrogen peroxide in 100 cc. of solution,

(13)	With 5 cc. of KOH solution (≡ 0.25 gm. of butyric acid)	yielded	0.00109	gm.	of	acetone.
(14)	With 5 cc. of NH_4OH solution (≡ 0.25 gm. of butyric acid)	"	0.00126	"	"	"
			0.00193	"	"	"
			0.00377	"	"	"
(15)	With 20 cc. of KOH solution	"	0.00092	"	"	"
(16)	" 20 " " NH_4OH "	"	0.00767	"	"	"
			0.00672	"	"	"
			0.0338	"	"	" *

* This result with the others in (16) show the extreme variations observed.

The results with (14) and (16) vary considerably with conditions, but can be duplicated for any set of conditions. Those for (13) and (15) are not subject to marked variations. Moreover, on standing longer than 2 days the results for (13) and (14) deviate farther and farther as the slow oxidation in (14) progresses. The final result in (14) after standing several weeks tends to approach the highest results obtained in (16). From this, one may conclude that the effect of ammonium hydroxide for a given time interval varies in the same way as the concentration and is in general influenced in the normal way by those factors that regulate the velocity of chemical reactions.

Interpretative Experiments.—In order to determine more accurately what happened in the above Experiments 15 and 16 with potassium and ammonium hydroxide, respectively, were repeated. After standing at room temperature nearly 3 days (16) was free from peroxide while (15) still contained unchanged hydrogen peroxide. The evolution of oxygen from both was clearly perceptible during the first day or more.

When (16) was analyzed by the method described above 0.0338 gm. of acetone or a 20.6 per cent yield was obtained. With 225 cc. or nine-elevenths of the distillate the Volatility Curve IV in Fig. 1 was obtained, which corresponds to a recovery of 0.1331 gm. or 52.4 per cent of the butyric acid used. The form of the curve shows that some lower fatty acid, probably acetic acid, was present. 0.219 gm. of barium carbonate, equivalent to 0.047 gm. of carbon dioxide or 4.7 per cent complete oxidation to carbon dioxide, was obtained. If it is assumed that all the carbon dioxide arises by oxidation of butyric acid to carbon dioxide and acetone thus: $CH_3CH_2CH_2CO_2H + O \rightarrow CH_3COCH_3 + CO_2 + H_2O$, then the carbon dioxide found corresponds to a yield of 18.8 per cent for this reaction.

The acetone obtained (20.6 per cent) and the butyric acid recovered (52.4 per cent) account for 73.0 per cent of the butyric acid used. The results indicate that much butyric acid was oxidized in the presence of ammonia.

When (15), containing potassium hydroxide instead of ammonium hydroxide, was analyzed in exactly the same way 0.00396 gm. of acetone or a 2.4 per cent yield was obtained. 0.018 gm. of carbon dioxide as barium carbonate was obtained, which corresponds to 1.8 per cent complete oxidation to carbon dioxide. With nine-elevenths of the distillate the Volatility Curve V, Fig. 1 was obtained which corresponds to a recovery of 0.242 gm. or 96.8 per cent of the butyric acid used. A small amount of lower acids probably acetic was also present as indicated by the slight deviations for Fractions 3 and 4.

The total recovery of butyric acid is $96.8 + 2.4 = 99.3$ per cent. The results indicate that almost no butyric acid was oxidized in the presence of potassium hydroxide.

From the two preceding experiments, one must conclude that ammonium butyrate is nearly 50 per cent oxidized under those conditions, while potassium butyrate is not appreciably attacked.

Influence of Ammonium Hydroxide in the Presence of Potassium Hydroxide.—As an interpretation of the results described above it might be suggested that in such dilute solutions the ammonium butyrate is largely hydrolyzed in comparison with the potassium butyrate and that this fact may give the key to the interpretation. In order to determine if this may be true some experi-

ments were done to test the influence of the simultaneous presence of potassium hydroxide. On this basis the amount of oxidation occurring could be expected to diminish as more potassium hydroxide was added.

Experiment 16 was repeated as follows:

(17). The same as Experiment 16 except that 5 cc. of potassium hydroxide (≡ 0.25 gm. of butyric acid) were added.

(18). The same as Experiment 16 except that 10 cc. of the same potassium hydroxide solution were added. This is double the amount of potassium hydroxide necessary to completely neutralize the butyric acid used.

The amount of ammonium hydroxide used was the same in both cases as in No. 16.

The solutions contained 1.50 per cent hydrogen peroxide at the beginning. After 92 hours No. 17 contained not over 0.06 per cent and No. 18 about 0.19 per cent hydrogen peroxide. Experiment 17 on analysis in the usual way gave 0.0239 gm. or a 14.6 per cent yield of acetone. Carbon dioxide corresponding to 0.052 gm. or a 5.2 per cent complete combustion to carbon dioxide was obtained. The fractionation of nine-elevenths of the total distillate gave the data for the unchanged butyric acid in Curve VI in Fig. 1, which corresponds to 0.152 gm. or a 60.8 per cent recovery of the butyric acid used.

No. 18 analyzed in the usual way gave 0.012 gm. of acetone or a 7.3 per cent yield. No carbon dioxide was evolved during the first distillation as with No. 20 or ammonium hydroxide experiments, but about a 6.0 per cent yield of carbon dioxide was obtained during the phosphoric acid distillation. The fractionation of four-fifths of the total distillate gave the data for the butyric acid in Curve VII, Fig. 1, which corresponds to 0.216 gm. or 86.4 per cent of the butyric acid recovered unchanged.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.
	per cent	*per cent*	*cc.*	*cc.*
15	2.4	96.8	20	0
18	7.3	86.4	10	20
17	14.6	60.8	5	20
16	20.0	52.4	0	20

These results both for acetone found and for butyric acid recovered are intermediate between those of Nos. 15 and 16 as shown in the tabulated data. They show conclusively that the amount of oxidation taking place is definitely related to the amount of potassium hydroxide present when the amount of ammonium hydroxide is considerable and constant.

Influence of $NaHCO_3$ on the NH_4OH Effect.—The question as to whether ammonium butyrate plays a special rôle in this reaction may be tested in another way also. In other experiments that will not be described here, it was found that the bicarbonates of potassium and sodium catalytically decompose hydrogen peroxide much faster than the hydroxides do, but not so fast as do the carbonates and ammonium hydroxide. If the effect of ammonium hydroxide is essentially due to this catalytic effect on peroxide, then considerable oxidation should take place even when the ammonium hydroxide in (16) is partly replaced with sodium bicarbonate. Three experiments were done.

(19). 5 cc. (0.25 gm.) butyric acid solution. 5 cc. (≡ 0.25 gm. butyric acid) ammonium hydroxide solution. 5 cc. sodium bicarbonate (0.028 gm. per cc. of solution). 50 cc. 3 per cent hydrogen peroxide.

(20). Same as (19) except that 10 cc. of sodium bicarbonate solution were added.

(21). Same as (19) except that no bicarbonate was used.

The results obtained on analyzing the mixtures 45 hours later are tabulated below.

No.	H_2O_2 remaining.	Acetone yield.		Butyric acid recovered.	
		gm.	*per cent*	*gm.*	*per cent*
19	0.16	0.0078	4.7	0.222	88.8
20	0.28	0.0078	4.7	0.245	97.8
21	1.52	0.018	11.1	0.211	84.4

These results show that although the peroxide is nearly completely decomposed in (19) and (20) there is even less oxidation than in (21) in which there was no excess of ammonium hydroxide nor any other compound catalytically active toward peroxide. Although only 58 per cent of the amount of bicarbonate, equivalent to the ammonia and butyric acid, was used in (19), peroxide

decomposition was nearly complete, but oxidation was very slight. This shows that the strong catalytic effect of ammonium hydroxide on peroxide is probably not the only characteristic of this compound necessary for catalyzing the oxidation of butyric acid. In this respect the behavior of ammonia seems to be specific in the same way that that of phosphate is in the case of the oxidation of glucose to carbon dioxide with hydrogen peroxide.[8] There is, however, one difference in the results as they stand and that is that the effect of ammonia can be increased in suitable mixtures with potassium hydroxide as shown in the next section, while nothing was tried that stimulates the phosphate effect similarly.

The Combined Effect of Ammonium and Potassium Hydroxide.—The interpretative experiments in the above section are defective as far as suggestiveness for biological conditions is concerned, because too much of these bases was used. The experiments described below were done in order to extend the above experiments to smaller amounts of the bases when used simultaneously. In these experiments 0.25 gm. butyric acid was used and enough potassium hydroxide to neutralize this acid (*i.e.*, 0.159 gm. of potassium hydroxide). In all cases 50 cc. of 2.3 per cent hydrogen peroxide were used. The total volume was 80 cc. The results upon analysis after 96 hours at room temperature are tabulated below.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.	H_2O_2 left.*
	per cent	*per cent*	*cc.*	*cc.*	*per cent*
22	4.0	88.0	5.0	0.0	0.908
23	17.0	57.6	5.0	5.0	0.153
24	15.0	61.6	5.0	10.0	0.151
25	11.8	68.4	5.0	20.0	0.187

* All contained 1.45 per cent H_2O_2 at the beginning.

More carbon dioxide was obtained in No. 23 than in any of the other experiments but the amount was small in all cases.

The above experiments were repeated using somewhat different amounts of alkali. Otherwise the conditions were the same.

[8] Witzemann, E. J., *J. Biol. Chem.*, 1920–21, xlv, 1.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.	H_2O used.	H_2O_2 left.*
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*per cent*
26	3.2	97.0	5.0	0.0	5.0	1.760
27	11.3	68.4	5.0	5.0	0.0	0.646
28	7.9	87.2	0.0	5.0	5.0	1.085

* All contained 1.77 per cent H_2O_2 at the beginning.

Because No. 27 contained more available alkali than the others the above experiments were repeated as follows and the results given below were obtained.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.	H_2O used.	H_2O_2 left.*
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*per cent*
29	2.3	91.2	10.0	0.0	0.0	1.214
30	14.8	62.8	5.0	5.0	0.0	0.293
31	12.8	67.2	0.0	10.0	0.0	0.658

* Nos. 29 to 31 contained 1.64 per cent H_2O_2 at the beginning.

The results of the three sets of experiments show: (1) That the loss in peroxide from the solution is greatest in those cases in which the most butyric acid disappeared. (2) That the presence of one equivalent of ammonium hydroxide is most favorable for oxidation when one equivalent of potassium hydroxide is also present. (3) That the presence of two equivalents of either base is not so favorable for oxidation or for peroxide decomposition as the presence of one equivalent of each. (4) That the effect of these bases is not proportional to the available alkali or the hydroxyl ion concentration.

The Relative Influence of Sodium Hydroxide upon Butyric Acid Oxidation.—In the above experiments potassium hydroxide came to be used as the alkali instead of sodium hydroxide without any reason or intention of discrimination. Normally, sodium salts are probably more abundant in biological conditions. Accordingly, it seemed desirable to do a few more experiments on the influence of sodium hydroxide upon this oxidation. In the six experiments given below 0.25 gm. of butyric acid, 50 cc. of 3.2 per cent hydrogen peroxide, and the alkali given in the table were allowed to react in a total volume of 100 cc. for 5 days at room temperature. The results are summarized in the table.

No.	Acetone found.	Butyric acid recovered.	NaOH used.	NH_4OH used.	KOH used.	H_2O used.	H_2O_2 left.
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*
32	22.7	45.2	0.0	10.0	0.0	35	0.340
33	24.8	48.4	0.0	5.0	5.0	35	0.192
34	23.0	60.0	5.0	5.0	0.0	35	0.170
35	16.2	58.0	5.0	5.0	5.0	30	0.153
36	17.9	59.2	10.0	5.0	0.0	30	0.102
37	15.0	61.6	0.0	5.0	10.0	30	0.229

Comparison of the results in (32), (33), and (34) shows that the sodium hydroxide is somewhat less active than the potassium hydroxide when used in conjunction with ammonium hydroxide. This is confirmed by the results in (36) and (37). Other unpublished experiments on the influence of these two bases on the spontaneous decomposition of peroxide show about the same degree of difference in their action, when used in the absence of ammonia.

The results in (34) in which one equivalent of all three bases was used stand between (36) and (37), which is to be expected if the small difference in their behavior is correct.

Biologically these results are of interest because they show that if under any conditions fatty acid oxidation in the organism resembles these oxidations either the potassium or sodium salt of mixtures would be attacked with about the same ease in the presence of ammonia.

The results in this section are of especial interest in another way because they show that the catalytic influence of ammonium, sodium, and potassium as the bases is just the reverse of their influence as the phosphates, upon the peroxide oxidation of a number of simple organic compounds. Further experiments upon mixtures will be done in developing these unpublished results on phosphates.

Partial Interpretation.—The experiments in the preceding section bring out a number of facts concerning the interpretation. (*a*) The ammonium and potassium butyrates here under consideration are the salts of a weak and a strong base, respectively, with a weak acid. At these dilutions there would be considerably more free butyric acid produced by hydrolysis when

ammonia only is used. That this free acid is not the only significant factor in the catalysis is shown by the fact that the simultaneous presence of both bases promotes the greatest oxidation. (*b*) That the mere presence of many butyrate ions does not determine the oxidation, is shown by the fact that in the presence of ammonium hydroxide alone, in which, of all the experiments, there is the smallest concentration of butyrate ions, the oxidation proceeds much faster than when potassium hydroxide only is used where the butyrate ion concentration is greatest. (*c*) That the butyrate molecules do not determine the oxidation is shown by the fact that oxidation is slowest where the concentration of these is greatest; namely, in the solutions containing potassium hydroxide alone. (*d*) That the capacity to decompose peroxide spontaneously may be a factor is suggested by the fact that potassium decomposes peroxide much more slowly than ammonium hydroxide. That this is not the sole factor is shown by the experiments with sodium bicarbonate which decomposes peroxide well but does not facilitate these oxidations. (*e*) The concentration of the hydroxyl ion has no definite relation to the velocity of the oxidation as observed with these three bases.

The known facts about these oxidations are not yet sufficient for a satisfactory interpretation. The statements given in the next two paragraphs suggest a partial interpretation.

The oxidation of butyric acid in the presence of ammonia described above may belong to the so called "coupled" or induced oxidations. The formation of a peroxide of ammonia may represent one phase of this coupling. That such a peroxide of ammonia may be obtained under suitable conditions was shown by Melikoff and Pissarjewski.[9] If this peroxide is the intermediate concerned it is much more unstable than the peroxide of potassium since when equal concentrations of the two bases are placed in solutions of hydrogen peroxide the ammonium hydroxide decomposes the peroxide much more rapidly than the potassium hydroxide does. The conceptions of an ideal catalyst and the theory of dislocation of Böeseken[10] offer a concrete partial interpretation of this part of the effect of ammonia. According to this

[9] Melikoff, P., and Pissarjewski, L., *Ber. chem. Ges.*, 1897, xxx, 3144.

[10] Böeseken, J., *Kon. Akad. Wetensch. Amst. Versl.*, 1914–5, xxiii, 291; *Rec. trav. chim. Pays-Bas*, 1920, xxxix, 622.

view of catalysis that catalyst will be best which has the greatest tendency to form the least stable complex with the substrate.

If it is granted that the data justify this much of an interpretation for the interaction of ammonia and peroxide they fail to indicate how such a complex activates the oxidation of butyric acid. Sweeping hypotheses on this subject would be undesirable, especially in view of the fact that equally anomalous data are already in hand showing that when these bases are used as phosphates the effects on the oxidation of butyric acid are quite different.

Possible Biological Significance of the Ammonia Effect. — The experiments showed that butyric acid, whether free or combined with sodium or potassium, is peculiarly susceptible to oxidation by peroxide when ammonia is also present, and that under these conditions relatively little carbon dioxide but much acetone is obtained. This high degree of acetone formation depends upon the simultaneous presence and the interaction of ammonia, peroxide, and butyric acid. Without entering into details it would seem that these three components play an active rôle in metabolism, particularly in the hepatic metabolism, and that acetone as acetoacetic acid is also formed in this organ. It will be recalled, for instance, that Embden and Kalberlah[11] found that the liver is the only organ that, on perfusion in the surviving state with normal blood, gives acetoacetic acid. Moreover there is in the liver an active traffic in ammonia equivalents involved in its important deaminizing and urea-forming functions. That it can actually supply this component of this reaction system better than other organs has recently been brought out by experiments of Keeton[12] of this laboratory, who found, in harmony with old observations, that when an inorganic acid is administered by stomach to dogs it is partially excreted as the ammonium salt, and causes an increase in the urinary ammonia nitrogen, both relative and absolute. When, however, the acid is injected into a peripheral vein, although it may cause some absolute increase of the urinary ammonia, it causes little or no increase in the quan-

[11] Embden, G., and Kalberlah, F., *Beitr. chem. Physiol. and Path.*, 1906, viii, 121. Embden, G., and Engel, H., *Beitr. chem. Physiol. u. Path.*, 1908, xi, 323.

[12] Keeton, R. W., *J. Biol. Chem.*, in press.

tity of ammonia nitrogen relative to the total nitrogen. This difference in the behavior of acid administered by the portal and the peripheral routes, respectively, is interpreted as due to the fact that ammonia is an available base in the liver and enters into the neutralization of acid when the acid is neutralized in the liver, but plays little part in the neutralization of acids elsewhere in the body.

It may not seem amiss to suggest, since the same type of oxidation as that observed in the above described test-tube experiments is known to occur in the liver in a high degree, that this oxidation in the liver may possibly be favored as in the test-tube by ammonia.

The question has long been discussed as to whether the formation of acetoacetic and β-hydroxybutyric acids in the body represents a purely abnormal type of fatty acid oxidation, that only occurs under certain conditions (as in diabetic acidosis, etc.) or whether it is a step in the normal oxidative breakdown of the fatty acids, having an even number of carbon atoms, which is obscured in health because of the rapid occurrence of subsequent events. The first view would make acetoacetic acid an abnormal product, the latter would make it a normal product, which in acidosis of the diabetic type fails to break down further. Neubauer[13] in his well known study of the breakdown of the fatty and amino-acids favored the view that the formation of acetoacetic acid is a normal step.

The above experiments and discussion would tend to favor the view that the formation of acetoacetic acid may be considered as a normal step in the oxidation of fatty acids, in harmony with Neubauer's conception, and particularly in media in which ammonia is available. Moreover the special occurrence of this type of oxidation in the liver might possibly be brought into relationship with the special availability of ammonia in this organ, it being less characteristic of the oxidation of fatty acids elsewhere in the body, where relatively less ammonia is available. According to this line of thought a certain special physiological function of the body, namely its power to form acetoacetic acid, would be associated with a special characteristic of ammonia

[13] Neubauer, O., *Deutsch. Arch. klin. Med.*, 1908–09, xcv, 211.

or its equivalent and the suggestion would follow that where acetoacetic acid is formed in the body ammonia may be present and acting in this characteristic way. The problem of why in health the evidence of acetoacetic acid formation is suppressed, *i.e.* the problem of "antiketogenesis" (or "ketolysis") is clearly another matter.

SUMMARY.

Experiments were performed with the object of determining the reason for the difference in the results obtained in the oxidation of butyric acid with hydrogen peroxide in the presence of different alkaline substances.

In the presence of potassium hydroxide in amounts varying from 0.20 to 4.0 equivalents no appreciable oxidation of butyric acid took place, as was proved by the almost quantitative recovery of the unchanged acid.

In the presence of ammonium hydroxide in amounts varying from 0.20 to 10.0 equivalents much oxidation took place.

The amount of oxidation in the presence of ammonia was found to increase with increase in the ammonium hydroxide, other things being equal, until more than 4.0 equivalents of ammonium hydroxide were present, after which it decreased somewhat.

This decrease with large excess of ammonium hydroxide was due to the spontaneous liberation of oxygen by the action of ammonium hydroxide on the hydrogen peroxide, before it could be utilized in oxidation.

If one equivalent of both ammonium hydroxide and potassium hydroxide is used more oxidation takes place than if two equivalents of either of these bases are added.

The type of oxidation observed in these experiments was mainly of the β type or the conversion of butyric acid into acetone and 1 molecule of carbon dioxide. Consequently the statement in the above paragraph is equivalent to saying that the presence of ammonia in a solution containing potassium butyrate and hydrogen peroxide catalyzes the oxidation of butyric acid to acetone and carbon dioxide.

The additive effect of ammonium and potassium here described in favoring oxidation constitutes a chemical analogy to the biological effects of certain mixed salts.

The experiments on the additive effects of sodium and ammonium show that this pair acts nearly the same as potassium and ammonium. There is just enough difference in the behavior of potassium and sodium to constitute a confirmation of unpublished observations of specific effects of these bases as phosphates.

A few suggestions for a partial interpretation were made. It is clear, however, in this case, as in the phosphate effect on glucose previously reported, that the rôle of alkalinity and alkali is not primary in this oxidation. A satisfactory interpretation is particularly desirable in view of the possible biological significance of the results.

Finally in discussing the possible biological significance of these results it is suggested that the ammonia effect here described may be the agency by which the normal oxidation of fatty acids is brought about in the liver. This suggestion rests upon the fact that in the liver the substances required for this effect are all available and that this organ also normally shows the greatest tendency to form acetoacetic acid.

ANTIKETOGENESIS.

III. CALCULATION OF THE KETOGENIC BALANCE FROM THE RESPIRATORY QUOTIENTS.

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In the first paper of this series (1) it was shown that the oxidation of glucose by hydrogen peroxide in alkaline solution brings about the rapid disappearance of acetoacetic acid if the latter be present; and this "ketolytic" reaction was described as an *in vitro* analogy to the well known "antiketogenic" effect of food carbohydrate in preventing or abolishing the appearance of the "acetone bodies" as metabolic end-products in man. In a second paper (2) the attempt was made to calculate the metabolic mixture of different subjects, in terms of the relative molecular amounts of all substances which are convertible in the body into acetoacetic acid and its related acetone and hydroxybutyric acid (ketogenic substances), and of all substances which are convertible into glucose and have an opposing, antiketogenic action. The calculation was based upon certain assumptions, some of which have experimental justification while others are of the nature of first trial guesses. The main assumptions are the following:

1. Each molecule of fat is convertible into (*a*) 3 molecules of acetoacetic acid and (*b*) 0.5 molecule of glucose, or its equivalent of antiketogenic derivative.

2. Protein is convertible, (*a*) into antiketogenic glucose or its equivalent to the extent of 3.6 gm. for each gm. of urine nitrogen and (*b*) into acetoacetic acid for each molecule of leucine, phenylalanine, and tyrosine, it being calculated that each gm. of urine nitrogen corresponds to approximately 10 millimols of ketogenic substance. (*c*) The amino-acids, valine, lysine, histidine, and tryptophane are neutral as to ketogenesis.

3. Carbohydrate exerts its antiketogenic action in the form of glucose or other hexose, 1 gm. of which is therefore equivalent to (1,000÷180=) 5.56 millimols of antiketogenic substance.

In the paper referred to, the calculation of the metabolic mixture was made on subjects under such conditions as to permit the additional assumptions, that the amount of carbohydrate burned was the amount fed, and that the fat catabolized was represented by the difference between the estimated total calories and the sum of the calories from carbohydrate and protein, the protein being indicated by the nitrogen excretion.

Such a calculation of the total ketogenic and antiketogenic substance in the metabolic mixture being oxidized by a number of different subjects appeared to show that definitely abnormal amounts of acetone bodies first appeared *when the molecular ratio of ketogenic to antiketogenic substance exceeded 1:1.* This fact is interpreted as indicating that the avoidance of the appearance of the acetone bodies is due to the "ketolytic" decomposition of acetoacetic acid as fast as it is formed, by its reaction with a product of glucose oxidation (or related substance from certain amino-acids and from glycerol), there being for this purpose nearly always in normal subjects on ordinary diets an abundance of glucose and other antiketogenic derivatives undergoing catabolism. Whenever the rate of production of ketolytic material falls below the rate of the catabolism of ketogenic substances as happens when the normal subject greatly reduces the carbohydrate intake, and in the diabetic when his power of metabolizing carbohydrate is sufficiently low, there is a deficit of ketolytic substance and in proportion to this deficit, acetoacetic acid accumulates, is in part converted into acetone and hydroxybutyric acid, and in the three forms is excreted as abnormal end-products.

According to this conception the starvation acidosis of any subject and the often more severe acidosis of diabetes are alike the result of, and in proportion to the unusual ratio between the rates of the catabolism of ketogenic substance on the one hand, and of the formation of the necessary ketolytic substances on the other.

It is the purpose of the present paper to describe another method of making a similar calculation of the metabolic mixture

and of the ketogenic-antiketogenic balance, from the respiratory exchange; and to show that this second method yields results which are in harmony with the conclusions above stated.

The significance of the respiratory quotient in metabolism experiments with man and animals is that it permits the calculation of the relative amounts of the foodstuffs being oxidized at a given time. In the Zuntz method, elaborated by Loewy (3) and extensively used by Lusk, Du Bois, Benedict, and others, the calculated amounts of oxygen and of carbon dioxide corresponding to the protein (the urine nitrogen), is subtracted from the total oxygen consumed and the total carbon dioxide exhaled; the remainders represent the metabolism of fat and carbohydrate and the ratio of the volumes of these gases is the "non-protein respiratory quotient" which by interpolation between the theoretical respiratory quotients indicates the proportion of fat to carbohydrate being burned. This method aims to divide the total metabolism into three fractions, of protein, fat, and carbohydrate. For our purposes it is necessary to divide the total somewhat differently; *viz*, into (*a*) *total* glucose (or equivalent antiketogenic substances) including glucose from amino-acids and from glycerol of fat, (*b*) fatty acid (ketogenic), and (*c*) the ketogenic fraction of protein.

The principle of our calculation is briefly as follows. It is assumed that in the normal subject as well as in the diabetic, glucose (or equivalent substance) is formed from protein to the extent of 3.6 gm. for each gm. of nitrogen and as such is to be included in the carbohydrate metabolism. The remainder of the protein, which may be called the non-carbohydrate quota of protein, is assumed to be oxidized parallel with the nitrogen excretion. While the latter assumption is perhaps not always correct, the error is less than results from the assumption that the *whole* of the protein is concurrently oxidized.

The oxygen and carbon dioxide corresponding to the non-carbohydrate quota of protein are subtracted from the total O_2 and CO_2; the remainders represent the oxidation of *total* glucose (from carbohydrate, protein, and glycerol) and of fatty acid. Their ratio, which is the "fatty acid-total glucose respiratory quotient," interpolated between the theoretical quotients for fatty acid and for glucose shows the relative participation of

fatty acid and glucose in the mixture being burned. Finally a correction is made for the ketogenic fraction of protein.

The details of the calculation are given below.

Protein Metabolism.—The calculation of Loewy (3) is taken as the basis, and from these data are subtracted the CO_2, O_2, and calories corresponding to the metabolism of the glucose derivable from protein.[1]

	C	H	O	N	Calories.
	gm.	*gm.*	*gm.*	*gm.*	
100 gm. of meat protein contain	52.38	7.27	22.68	16.65	563.09
Of which there appear in urine	9.406	2.663	14.099	16.28	131.46
" " " " " feces	1.471	0.212	0.889	0.37	
Remainder oxidized in body	41.50	4.40	7.69	0	

With production of..152.17 gm. CO_2, 39.6 gm. H_2O, and 431.63 calories.
Which requires......138.18 gm. additional O_2
16.28 gm. of urine nitrogen are thus equivalent to
152.17 gm. CO_2 = (× 0.5089) = 77.437 liters CO_2
138.18 gm. O_2 = (× 0.6998) = 96.694 liters O_2

	CO_2		O_2		Calories.	R. Q.
	gm.	*liters*	*gm.*	*liters*		
or 1 gm. of urine nitrogen is equivalent to	9.47	4.757	8.488	5.939	26.51	0.801
From 3.6 gm. of glucose	5.28	2.687	3.84	2.687	13.54	1.000
Non-carbohydrate quota of protein for 1 gm. of urine N	4.067	2.07	4.648	3.252	12.97	0.6365

The values given in the last line, multiplied by the nitrogen excretion during the period, are subtracted from the respective respiratory data, and the ratio of the remainders of CO_2 and O_2 is the "fat-total glucose respiratory quotient." One may de-

[1] This step in the calculation, the correction for the non-carbohydrate quota of protein, is substantially that described by Magnus-Levy (4) and by Lusk (5) for the theoretical non-protein respiratory quotient in "total" diabetes. It is applicable, however, to data from any subject. A few of our data differ very slightly from those of Loewy and Lusk because of the choice of slightly different values for the weights of a liter of CO_2 and O_2, and for the heat of combustion of glucose.

termine from this quotient the percentage of calories from fat and of calories from carbohydrate by reference to the table prepared by Lusk (6)[2] or the relative amounts (in calories) of glucose (including glycerol) and of *fatty acids*, and the *molecular ratio of their mixtures* may be learned from Table II.

The derivation of the data in this table requires explanation. The oxidation of 1 gm. molecule (180 gm.) of glucose, the respiratory quotient for which is 1.000 requires 192 gm. of O_2 and produces 264 gm. of CO_2 and $3.762 \times 180 = 677.2$ calories. The caloric value of oxygen for the oxidation of glucose (at R. Q. of 1.000) is therefore:

$$1 \text{ liter of } O_2 = \frac{677.2}{192 \times 0.6998} = 5.040 \text{ calories.}$$

Fatty Acid.—We have assumed a certain arbitrary mixture of olein, palmitin, and stearin as representing a probable average body fat and from this have calculated the respiratory quotient of the contained fatty acids, in order to exclude the glycerol.[3]

[2] Used in this way, the above procedure has the advantage over the usual method of calculating the "non protein R.Q." in that it avoids the erroneous assumption that all of the carbon of protein is oxidized concurrently with the excretion of nitrogen. Not only is it advantageous in severe diabetes, when a large part of the protein is not oxidized, and for which Lusk uses a special method of calculation, but in also many other circumstances, well illustrated by the high protein experiments of Williams, Riche, and Lusk (8) in which the protein calories corresponding to the nitrogen excretion even exceeded the total heat produced.

[3] Although it is not known at what stage glycerol is split off from the fatty acids, it seems probable that the split occurs before the oxidation of the carbon atom beta to the carboxyl. When the split occurs it is likely that the glycerol is oxidized to a substance which is either condensed to glucose or further oxidized. Free glycerol is known to be convertible into glucose in the body, and although direct evidence is lacking as to the fate of glycerol in the form of esters or glycerophosphoric acid, it is for the present assumed that it takes the same course. If, as is commonly supposed, the phosphatides represent a stage in the catabolism of fats, the possibility is suggested that it is the third molecule of fatty acid, replaced by phosphoric acid, which is first oxidized, and that final oxidation of the others occurs only as they are split off from the phosphatide molecule.

	C	H	O
Olein, $C_{57}H_{104}O_6$, 70 molecules................	3,990	7,280	420
Palmitin, $C_{51}H_{98}O_6$, 15 molecules.............	765	1,470	90
Stearin, $C_{57}H_{110}O_6$, 15 molecules..............	855	1,650	90
Sum, 100 molecules..........................	5,610	10,400	600
Multiply by atomic weights....................	67,320	10,483	9,600

Average molecular weight = 874

Of average percentage composition, 77 per cent C, 11.9 per cent H, 11.1 per cent O

The respiratory quotient for the *complete oxidation* of this mixed fat would be as follows:

$$C_{56.1}H_{104}O_6 + 79.1\ O_2 = 56.1\ CO_2 + 52\ H_2O$$

$$\frac{56.1\ CO_2}{79.1\ \ O_2} = 0.709$$

874 gm. (1 gm. molecule) of such a mixed fat would yield

$56.1 \times 44 = 2,468.4$ gm. of CO_2 and would require $79.1 \times 32 = 2,531.2$ gm. of O_2; or 1 gm. of fat would be equivalent to 2.824 gm. of CO_2 and to 2.896 gm. of O_2. These values are very similar to those of actual analyses as shown in Table I.

TABLE I.

	C	H	O	Oxidation of 1 gm. of fat.		R. Q
				CO_2	O_2	
	per cent	*per cent*	*per cent*	*gm.*	*gm.*	
Lard*................	76.5	11.9	11.6	2.805	2.876	0.710
" †................						0.707
Human fat‡..........	76.1	11.8	12.1	2.790	2.845	0.713
Mixed fat............	77.0	11.9	11.1	2.824	2.896	0.709

* Lehmann, C., Mueller, F., Munk, I., Senator, H., and Zuntz, N., *Virchows Arch. path. Anat.*, 1893, cxxxi, suppl., 131.

† Zuntz, N., *Arch. ges. Physiol.*, 1897, lxviii, 201.

‡ Zuntz, N., and Loewy, A., Lehrbuch der Physiologie des Menschen, Leipsic, 2nd edition, 1913, 644.

But if this mixed fat be *converted into fatty acid and glucose* (from glycerol) the following amounts would be formed.

	C	H	O
From 100 molecules of fat:			
70 mol olein → 210 mol oleic acid ($C_{18}H_{34}O_2$)......................	3,780	7,140	420
15 mol palmitin → 45 mol palmitic acid ($C_{16}H_{32}O_2$)......................	720	1,440	90
15 mol stearin → 45 mol stearic acid ($C_{18}H_{36}O_2$)......................	810	1,620	90
300 mol fatty acid, average = $C_{17.7}H_{34}O_2$ molecular weight 278......................	5,310	10,200	600
50 mol glucose......................	300	600	300
	5,610	10,800	900
In original fat......................	5,610	10,400	600
Added......................		400	300
As 200 mol H_2O......................		400	200
As 50 mol O_2......................			100

The reaction may be written as follows:

(1) 100 mols fat + 300 mols H_2O + 50 mols O_2 → 50 mols glucose
(100 × 874 gm.) (50 × 180 gm.)
+ 300 mols fatty acid + 100 mols H_2O
(300 × 278 gm.)

If the fatty acid be then oxidized,

(2) 300 mols fatty acid + 7,560 mols O_2 → 5,310 mols CO_2 + 5,100 mols H_2O

The respiratory quotient for the complete reaction, *oxidizing the fatty acids and converting the glycerol into glucose,* would be

$$\frac{5{,}310\ CO_2}{7{,}560 + 50\ O_2} = 0.698$$

In the above reaction the heat liberated is

874 gm. fat × 9.461 calories (3)	=	8,269 calories
Deduct 90 gm. glucose	=	338.6
		7,930.4

The caloric value of a liter of oxygen, *when the fatty acid of fat is oxidized and the glycerol converted to glucose* (at R.Q. 0.698) is therefore

$$\frac{7{,}930}{(75.6 + 0.5) \times 32 \text{ gm.} \times 0.6998} = 4.653$$

It may be further calculated that *in undergoing this reaction* 1 gm. of fat (containing 278 × 3 ÷ 874 = 0.9542 gm. of fatty acid) liberates 7,930 ÷ 874 = 9.074 calories and accordingly 1 gm of *fatty acid* furnishes 9.509 calories.[4]

[4] This figure includes also the heat involved in the hydrolysis of the glyceride and in the conversion of glycerol to glucose.

TABLE II.

Respiratory Quotients for Total Glucose and Fatty Acid.

From the total CO_2 and O_2 subtract amounts corresponding to the metabolism of the non-carbohydrate quota of protein during the respiration period.

	CO_2		O_2		Calories.
	gm.	*liters*	*gm.*	*liters*	
Non-CH quota of protein for 1 gm. of urine N.	4.067	2.07	4.648	3.252	12.97
logs	= .60927	.31597	.66727	.51215	.11294
	1 gm. = 0.5089 liter log = .70662		1 gm. = 0.6998 liter log = .84497		

The remainders represent the metabolism of fatty acid and glucose and their ratio (in liters) is the "fatty acid: glucose respiratory quotient."

Fatty acid: glucose respiratory quotient.	Caloric value of 1 liter of O_2.		Per cent of calories from		Molecular ratio of the mixture burned. $\frac{\text{Fatty acid}}{\text{Glucose}}$
	Calories.	Logarithm.	Glucose.	Fatty acid.	
(1)	(2)	(3)	(4)	(5)	(6)
0.698	4.653	66777	0	100.0	
0.70	4.656	66801	0.7	99.3	36.8
0.71	4.669	66922	4.0	96.0	6.2
0.72	4.682	67043	7.3	92.7	3.2
0.73	4.695	67164	10.6	89.4	2.17
0.74	4.707	67274	13.9	86.1	1.6
0.75	4.720	67394	17.2	82.8	1.24
0.76	4.733	67514	20.5	79.5	1.01
0.77	4.746	67633	23.8	76.2	0.83
0.78	4.758	67742	27.1	72.9	0.70
0.79	4.771	67861	30.4	69.6	0.60
0.80	4.784	67979	33.6	66.4	0.52
0.81	4.797	68097	37.0	63.0	0.44
0.82	4.810	68215	40.4	59.6	0.39
0.83	4.823	68332	43.7	56.3	0.34
0.84	4.835	68440	47.0	53.0	0.28
0.85	4.848	68556	50.3	49.7	0.25
0.86	4.861	68673	53.6	46.4	0.22
0.87	4.874	68789	56.9	43.1	0.19
0.88	4.887	68904	60.2	39.8	0.17
0.89	4.899	69011	63.5	36.5	0.15
0.90	4.912	69126	66.8	33.2	0.13
0.91	4.925	69241	70.2	29.8	0.11

TABLE II—*Concluded.*

Fatty acid: glucose respiratory quotient.	Caloric value of 1 liter of O_2.		Per cent of calories from		Molecular ratio of the mixture burned. $\frac{\text{Fatty acid}}{\text{Glucose}}$
	Calories.	Logarithm.	Glucose.	Fatty acid.	
(1)	(2)	(3)	(4)	(5)	(6)
0.92	4.938	69355	73.5	26.5	0.093
0.93	4.950	69461	76.8	23.2	0.078
0.94	4.963	69574	80.1	19.9	0.065
0.95	4.976	69688	83.4	16.6	0.052
0.96	4.989	69801	86.7	13.3	0.039
0.97	5.001	69906	90.0	10.0	0.029
0.98	5.014	70018	93.3	6.7	0.019
0.99	5.027	70131	96.7	3.3	0.009
1.00	5.040	70245	100.0	0.0	

The data above calculated for the theoretical respiratory quotients of fatty acid and of glucose and the corresponding caloric values of oxygen are given in Table II, together with interpolated values between the extremes. Corresponding to each quotient, in Columns 4 and 5 are given the percentage of the total fat-glucose calories derived from glucose and from fatty acid.

From the latter figures one obtains the relative *molecular* amounts of each, that is their molecular ratio, by multiplying each percentage, by the corresponding fraction of a molecule of fatty acid and of glucose which is equivalent to 1 calorie, and dividing the product for fatty acid by the product for total glucose. In accordance with the assumption that each molecule of fatty acid gives rise to 1 molecule of acetoacetic acid, and that each molecule of glucose is equivalent to 1 of antiketogenic substance, the ratio as above obtained is the ketogenic-antiketogenic ratio (Column 6) except that it does *not include the ketogenic* value of protein. The latter, however, may be included very simply, since the ketogenic value corresponding to 1 calorie from protein proves to be almost exactly the same as the ketogenic value corresponding to 1 calorie from fatty acid; and the percentage of calories from (total) protein may therefore be added and calculated with the fatty acid calories. The values in question are stated below.

	Calories.	Ketogenic millimols.	Anti-ketogenic millimols (as glucose).
Protein, 1 gm. urine nitrogen =	26.5	10	20
Each calorie from protein =		0.377	0.755
Fatty acid, 1 gm. =	9.509	3.6	0
Each calorie from fatty acid =		0.378	0
Glucose, 1 gm. =	3.76	0	5.56
Each calorie from glucose =		0	1.478

$$\frac{\begin{pmatrix}\text{Per cent of calories from} \\ \text{fatty acid + per cent of} \\ \text{calories from protein}\end{pmatrix} \times \begin{pmatrix}\text{Ketogenic value of 1} \\ \text{F. A. (or protein)} \\ \text{calorie} = (0.377)\end{pmatrix}}{\begin{pmatrix}\text{Per cent of calories from} \\ \text{total glucose}\end{pmatrix} \times \begin{pmatrix}\text{Antiketogenic value} \\ \text{of 1 glucose calorie} \\ = (1.478)\end{pmatrix}} = \frac{\text{Ketogenic}}{\text{Antiketogenic}}\ \text{ratio.}$$

It is convenient to treat the second factors in numerator and denominator separately as a single fraction, $\frac{0.377}{1.478} = 0.255$.

As examples of the complete calculation of data of individual experiments the following may be given.

(1) Urinary nitrogen excretion per minute = 12.7 mg.

	CO_2	O_2	R. Q.
	cc.	*cc.*	
Total per minute	232	312	0.745
12.7 mg. N × 2.07 cc. CO_2	26.3		
12.7 mg. N × 3.25 cc. O_2		41.3	
From fat and C H, including glucose from protein	205.7	270.7	0.760

0.271 liters O_2 × 4.733 calories (at R.Q. 0.76) . . = 1.284 calories per minute from fat and C H, including glucose from protein.

Non-C H quota of protein, 0.0127 gm. N per minute × 12.97 = 0.165 calories

Total 1.449 calories per minute

0.0127 gm. × 26.5 = 0.336 calories from *total* protein.

$\frac{0.336}{1.449} \times 100 \quad = 23.2$ per cent of total calories from protein.

0.760 "F.A.-G. R. Q." corresponds to 79.5 per cent of F.A.-G. calories from fatty acid, and 20.5 per cent of F.A.-G. calories from total glucose.

$$\frac{79.5 + 23.2}{20.5} \times 0.255 = 1.28 \text{ ketogenic ratio.}$$

(2) Urinary total nitrogen per minute = 14.2 mg.

	CO_2	O_2	R. Q.
Total, per minute	246	324	0.765
14.2 mg. N × 2.07 cc. CO_2	29.4		
14.2 mg. N × 3.25 cc. O_2		46.2	
From fat and C H, including glucose from protein	216.6	277.8	0.780

278 liters O_2 × 4.758 calories (at R.Q. 0.78) = 1.323
0.0142 gm. N × 12.97 calories = 0.184
Total calories per minute = 1.507
0.0142 × 26.5 = 0.376 calories from total protein.

$\frac{0.376}{1.507} \times 100 \quad = 24.9$ per cent from total protein.

0.780 F.A.-G.R.Q. indicates 72.9 per cent fatty acid calories and 27.1 per cent total glucose calories.

$$\frac{72.9 + 24.9}{27.1} \times 0.255 = 0.92 \text{ ketogenic ratio.}$$

The above calculation, which is rather tedious when applied to many data, is fortunately not necessary to obtain the information which it yields. It so happens that the two corrections involved; (*a*) the increase in the R. Q. from the glucose quota of protein and the consequent effect in lowering the ratio, and (*b*) the effect of the ketogenic influence of protein in raising the ratio, although not equal, are in opposite directions, and one may get almost the same result by ignoring the protein and calculating the *total* R. Q. as though only fatty acid and glucose were being burned. In this way almost all calculation is avoided. In Table III the ratios so obtained from the total R. Q. (in Column 2) are compared with the ratios corrected as above illustrated for both effects of protein, when the protein metabolism amounts to 10, 15, 20, and 25 per cent of the total energy exchange. From this comparison it is evident that, considering the technical difficulty

in exact and reliable determinations of respiratory quotients, the ketogenic ratios obtained directly from the total respiratory quotients are probably as nearly correct as can be expected from data of this character. We shall, therefore, in the following analysis of experimental data use only the total R. Q.; although it may be stated that we have also made the fully corrected calculations on nearly all of the data presented.

Perhaps it may be also explained that while the foregoing somewhat lengthy method of calculation of the ketogenic ratio from respiratory data is being abandoned as unnecessary, the short cut is permissible only because of the longer analysis of the underlying considerations.

TABLE III.

Ketogenic Ratios and Respiratory Quotients at Different Levels of Protein Metabolism.

Total R. Q.	Ketogenic ratio, Column 6, Table I.	10 per cent protein calories.		15 per cent protein calories.		20 per cent protein calories.		25 per cent protein calories.	
		F.A.-G. R.Q.	Corrected ratio $\frac{K}{A}$	F.A.-G. R.Q.	Ratio $\frac{K}{A}$	F.A.-G. R.Q.	Ratio $\frac{K}{A}$	F.A.-G. R.Q.	Ratio $\frac{K}{A}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
0.72	3.2	0.726	2.7	0.728	2.65	0.731	2.6	0.734	2.4
0.73	2.17	0.736	1.96	0.739	1.9	0.742	1.85	0.746	1.78
0.74	1.6	0.746	1.5	0.750	1.44	0.753	1.43	0.756	1.42
0.75	1.24	0.757	1.18	0.761	1.18	0.765	1.12	0.768	1.14
0.76	1.01	0.767	0.98	0.772	0.94	0.777	0.93	0.780	0.92
0.77	0.83	0.778	0.82	0.782	0.80	0.788	0.78	0.791	0.80
0.78	0.70	0.788	0.70	0.792	0.70	0.799	0.68	0.802	0.69
0.79	0.60	0.80	0.59	0.804	0.59	0.810	0.57	0.813	0.60
0.80	0.52	0.811	0.5	0.816	0.50	0.821	0.5	0.827	0.50
0.81	0.44	0.821	0.43	0.826	0.44	0.832	0.43	0.838	0.44

According to the data in Table III *a total respiratory quotient of 0.76 indicates the oxidation of a metabolic mixture made up of approximately equimolecular amounts of ketogenic substances (fatty acids or ketogenic amino-acids), and of antiketogenic derivatives of amino-acids, glycerol, or carbohydrate, expressed in terms of glucose.* Expressed in the same terms, a respiratory quotient of 0.73 indicates approximately 2 molecules of ketogenic to 1

of antiketogenic substance in the mixture; while a quotient of 0.80 indicates only 0.5 molecule of ketogenic to 1 of antiketogenic glucose equivalent.

With these values in mind we may inspect any respiratory data and provided we assume that the latter are truly representative of metabolic reactions, the ketogenic ratio of the subject may be determined. The question we have attempted to answer is: What is the ratio at the time when definitely abnormal but *small* amounts of acetone bodies *first appear* in blood, urine, or breath?

After an examination of many experiments by others as well as by ourselves there appears to be no doubt that the mixture in all subjects, *at the threshold of ketonuria* is, according to respiratory data, that which corresponds to *equimolecular* mixtures of ketogenic and antiketogenic substances, *the latter being calculated in terms of glucose equivalents.* This is the same conclusion as that reached by a different method of analysis described in the earlier paper.

In the application of the foregoing calculation to experimental results it is essential that the respiratory data be accepted as reliable and as indicating the actual oxidation in the tissues; and our experience in this laboratory has fully impressed us with the dangers in assuming that this is so. A large number of observations with both Benedict unit spirometer apparatus and with large spirometers and gas analysis have been made on normal subjects by the writer and associates, and on hospital patients by my colleague, Dr. Olmsted, and while many of our results are consistent and probably correct, the R. Q. often varies in consecutive periods and for that reason is perhaps questionable; and it seems preferable to choose for this first analysis results from other laboratories, the accuracy and reliability of which are least open to question. Our own experiments will be reported in later papers.

Fasting Normal Subject "L" of F. G. Benedict (9).

The observations on this subject from the Carnegie Nutrition Laboratory are well known and require no comment. In Table IV are given the various total respiratory quotients of the subject, before, during, and immediately after the long fast. On the 2nd fast day the subject excreted 0.5 gm. of hydroxybutyric acid, and on the following days the amount varied from 1.4 to

7.0 gm. On the 1st and 2nd days after the resumption of food 0.8 and 0.5 gm. were excreted. The 1st and 2nd days of fast and the days after taking food may therefore be taken as the border-line of ketosis. It is evident from the table that acetone bodies first appeared when the R. Q. reached 0.75 or 0.76, and were disappearing when, after food, it reached 0.79 or 0.80. A respiratory quotient of 0.76 corresponds to a ketogenic ratio of about

TABLE IV.

Respiratory Quotients and Ketogenic Ratios of Fasting Subject "L."

F. G. Benedict (9).

Date.	Day.	Total R. Q. Table 50, p. 345.		Average R. Q.	Total R. Q. average for whole day, p. 396.	Ketogenic ratio $\frac{K}{A}$	Hydroxy-butyric acid determined. Table 38, p. 283.
		Bed calorimeter, night.	Respiratory apparatus, morning.				
Apr. 10–11	Food.	0.81	0.81	0.81		0.4	
" 11–12	"	0.88	0.89	0.885		0.16	
" 12–13	"	0.86	0.89	0.875		0.18	
" 13–14	"	0.81	0.82	0.815		0.4	
" 15	Fast 1	0.78	0.78	0.78	0.765	0.9	
" 16	" 2	0.75	0.79	0.77	0.755	1.4	0.5
" 17	" 3	0.73	0.75	0.74	0.748	1.5	2.1
" 18	" 4	0.74	0.75	0.745	0.73	2.2	3.5
" 19	" 5	0.75	0.77	0.76	0.72	3.0	2.1
" 20	" 6	0.68	0.74	0.71	0.72	3.0	3.5
" 21	" 7	0.71	0.75	0.73	0.73	2.2	2.8
" 22	" 8	0.73	0.74	0.735	0.734	1.9	1.6
" 23	" 9	0.75	0.75	0.75	0.725	2.7	3.5
" 24	" 10	0.72	0.76	0.74	0.726	2.7	3.5
" 25	" 11	0.72	0.75	0.735	0.733	1.8	1.4
May 13–14	" 30	0.72	0.72	0.72		3.0	5.4
" 14–15	" 31	0.72	0.72	0.72		3.0	4.5
	Food.						
" 16–17	1	0.80	0.78	0.79		0.6	0.8
" 17–18	2	0.97	0.94	0.96		0.03	0.5

1:1. As one would expect, the quotients fluctuate somewhat during each day, and assuming that they are "metabolic quotients" the ketogenic ratio of the metabolic mixture doubtless also fluctuates; but it is clear that acetone bodies first appeared when the quotient first dropped to 0.76 or below.

Normal Subjects on Non-Carbohydrate Diets (Higgins, Peabody, and Fitz, 10).

During 4 days each subject ate large amounts of protein and fat but no carbohydrate. On the 1st diet day small amounts of acetone bodies appeared in the urine. Respiratory metabolism was determined twice daily by spirometer and gas analysis. Table V contains a comparison of the total respiratory quotients, the corresponding ketogenic ratios, and the total acetone body excretion expressed as hydroxybutyric acid per day, for two of the subjects. In each case the afternoon R. Q. of the 1st diet days (0.745 and 0.715) correspond to ketogenic ratios greater than 1, after which time the quotients indicate much higher ratios, in general agreement with the large excretion of hydroxybutyric acid. The conclusion appears justified that both subjects first excreted acetone bodies when the ketogenic ratio exceeded 1.

Subject "Mrs. MCK" of Means (11), and Folin and Denis (12).

A very obese woman was observed during three fasts, the respiratory data being reported by Means and the urine analyses by Folin and Denis. The data essential to our discussion are brought together in Table VI.

During the diet period preceding the first fast and during that fast the respiratory quotients are low (0.74 and from 0.71 to 0.68), indicating ketogenic ratios higher then 1 even before the fast, when presumably there was no ketonuria. Also during the next diet period the R. Q. remained low in spite of much food carbohydrate (148 to 239 gm. per day), being 0.74 to 0.76. The same, or lower, quotients were found in each of the next two diet periods and are difficult to explain.

However, the quotients in both the second and third fast periods are wholly in accord with results from the preceding subjects. Acetone bodies first appeared when the quotient dropped below 0.76 indicating a ketogenic balance greater than 1.

Diabetic Case No. 740 of Joslin (13).

This case of severe diabetes with marked acidosis has been reported as an example showing "that if a diabetic is fasted, acidosis disappears and this is in marked contrast to the behavior

TABLE V.

Respiratory Quotients and Ketogenic Ratios.

Normal subjects on high protein, high fat, non-carbohydrate diet (Higgins, Peabody, and Fitz, 10).

Subject.	Date.	Respiratory period.	Total R. Q.	Ketogenic ratio. From total R. Q.	Ketogenic ratio. Calculated with corrections.	Total hydroxy-butyric acid in 24 hours.	Diet.
						gm.	
H. L. H.	June 1	8 a.m.	0.82	0.4	0 4		Mixed.
		4 p.m.	0.86	0.2	0.2		
	" 2	8 a.m.	0.82	0.4	0.3		Non-C H.
		4 p.m.	0.745	1.4	1.3	2.22	
	" 3	8 a.m.	0.73	2.1	1.4		" "
		4 p.m.	0.74	1.6	1.3	5.14	
	" 4	8 a.m.	0.725	2.6	1.8		" "
		4 p.m.	0.685	∞		18.3	
	" 5	8 a.m.	0 705	15.0			" " + 100 cc. whiskey.
		4 p.m.	0 700	40.0		25.6	
	" 6	8 a.m.	0.680	∞		3.4*	Mixed, 468 gm. C H.
		4 p.m.	0.765	0.9		1.85†	
F. W. P.	" 7	8 a.m.	0.82	0.4			
		4 p.m.	0.785	0.7			Mixed.
	" 8	8 a.m.	0.835	0.3			Non-C H.
		4 p.m.	0.715	4.0		0.93	
	" 9	8 a.m.	0.730	2.1			" "
		4 p.m.	0.700	40.0		2.6	
	" 10	8 a.m.	0.740	1.6			" "
		4 p.m.	0.675	∞		7.3	
	" 11	8 a.m.	0.725	2.5			" " + 180 cc. whiskey.
		4 p.m.	0.670	∞		15.5	
	" 12	8 a.m.	0.680	∞		1.1‡	Mixed, 386 gm. C H.
		4 p.m.	0.690	∞		1.0§	

* for 2.5 hrs.
† for 21.5 hrs.
‡ for 2 hrs.
§ for 22 hrs.

of normal men, for they present acidosis upon fasting with no increase in the respiratory quotient as the fasting proceeds."

TABLE VI.

Respiratory Quotients and Ketogenic Ratios.

Subject, Mrs. MCK. from Means (11), and Folin and Denis (12).

Date.	Total R. Q. average (Means).	Ketogenic ratio $\frac{K}{A}$	Total hydroxybutyric acid in 24 hours.	
			gm.	
Feb. 23	0.74	1.6		Food.
" 24	0.74	1.6		
" 25	0.71	6.0	0.34	First fast.
" 26	0.69		4.68	
" 27	0.69		20.72	
" 28	0.68		24.63	
Mar. 1	0.74	1.6		Food.
" 2	0.76	1.0		
" 3	0.76	1.0		
" 4	0.74	1.6		
" 5	0.75	1.2		
" 6	0.75	1.2		
" 7	0.76	1.0		
" 9	0.75	1.2		
" 10	0.76	1.0	0	Second fast.
" 11	0.75	1.2	0.03	
" 12	0.75	1.2	2.68	
" 13	0.72	3.0	8.60	
" 14	0.71	6.0	17.34	
" 15	0.73	2.1		Food.
" 16	0.73	2.1		
" 17	0.73	2.1		
" 19	0.78	0.7		
" 20	0.79	0.6	0	Third fast.
" 21	0.77	0.8	0	
" 22	0.74	1.6	3.47	
" 23	0.72	3.0	20.09	
" 24	0.71	6.0		Food.
" 25	0.75	1.2		
" 26	0.79	0.6		

Since it is the writer's contention that as regards the amount of acetone bodies formed the diabetic behaves essentially the same as any other subject who metabolizes the same mixture, it is

desirable that we attempt an analysis of this case. Table VII contains the total respiratory quotients, the corresponding ketogenic ratios, and the amount of total acetone bodies, expressed as hydroxybutyric acid, (including acetone and acetoacetic acid but not including breath acetone) which was actually excreted. Although as shown by a detailed calculation and as noted by Joslin the R. Q. is usually somewhat higher than is to be explained by the materials known to be available to the subject, if one assumes that the data represent metabolic quotients, it is evident that the acidosis decreased (but did not *disappear*) as the ketogenic ratio approached 1. According to the results from other subjects the continued excretion of about 5 gm. of hydroxybutyric acid per day is hardly to be expected with a respiratory quotient of 0.76 and suggests that the quotients are actually slightly higher than the true metabolic quotients, or that the quotients during the respiration periods were higher than at other periods of the day. With an existing acidosis, respiratory quotients are apt to fluctuate and their interpretation is difficult and uncertain. Whatever the explanation of this discrepancy, it is evident from an analysis of Joslin's data (not included in our table) that the gradual rise of the respiratory quotient, the lowering of the ketogenic ratio which that rise indicates, and the decline of the acidosis, were not caused by

TABLE VII.

Severe Diabetic with Decreasing Acidosis on Fasting.

Joslin, Case 740 (13).

Date.	Total R. Q.	Ketogenic ratio.	Total hydroxybutyric acid excreted
			gm.
Apr. 15-16	0.72	3.2	24.9
" 16-17	0.73	2.1	18.9
" 17-18	0.72	3.2	11.8
" 18-19	0.735	1.9	11.0
" 19-20	0.755	1.0	7.9
" 21-22	0.75	1.2	6.5
" 24-25	0.736	1.9	5.4
" 27-28	0.745	1.4	4.7
May 1	0.76	1.0	4.1

any increase in sugar burning power but were caused by the decrease in total metabolism resulting from the fast, and in the amount of ketogenic material (fat and protein) in the metabolic mixture. The metabolism of ketogenic substances was merely slowed down by undernutrition to the point where it no longer markedly overbalanced the already low rate at which the body was able to provide antiketogenic substance for "neutralization." This appears to the writer to be the probable explanation of the beneficial effect of fasting and undernutrition in causing a decrease of diabetic acidosis. The point of view will be developed in a later paper.

There are many other results from diabetics in the literature which are more or less suitable for similar examination and we have studied a number of them, especially in the publications of Benedict and Joslin from the Nutrition Laboratory and of Du Bois and associates from the Sage Laboratory at Bellevue Hospital. It is evident from these and earlier results as has been repeatedly noted, that the respiratory quotients are lower the more severe the grade of diabetes. The quotients determined by even the most expert investigators, however, often vary considerably from hour to hour and their interpretation is difficult. Moreover, most of the cases on which sufficient data are given had more or less severe acidosis and under such circumstances, factors resulting from acid production may so affect the respiratory quotient as to make it an unfavorable basis for calculating the ketogenic balance. A detailed consideration of other individual cases will accordingly not be attempted at present.

Attention may be called, however, to the summary of Benedict and Joslin (14) which shows (p. 113) that the average quotients of all of their observations on severe diabetics, with marked acidosis, was 0.73 *which corresponds to a ketogenic ratio of about 2*, while the average quotient of the moderately severe and mild diabetics *with little or no acidosis* was from 0.73 to 0.77, the latter *value corresponding to a ketogenic ratio of about 0.8*. This general finding is, therefore, quite in harmony with the conclusion that a R. Q. of 0.76, corresponding to a ketogenic ratio of 1 represents the border-line or threshold of ketosis.

SUMMARY AND CONCLUSIONS.

A method is described by which the ratio of ketogenic to antiketogenic molecules in the metabolic mixture of a subject may be calculated from the respiratory quotient.

Evidence is presented which appears to point to the conclusion that a molecular ratio of 1:1, which corresponds (according to the method of calculation) to a respiratory quotient of 0.76, is the limit for the avoidance of the excretion of acetone bodies. With quotients higher than 0.76 the catabolism of the antiketogenic glucose (or its equivalent from protein and glycerol) is great enough to remove acetoacetic acid as fast as it is formed, presumably by a "ketolytic" reaction analogous to the *in vitro* reaction previously described.

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13. Joslin, E. P., *Am. J. Med. Sc.*, 1915, cl, 490.
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OBSERVATIONS ON THE "ALKALINE TIDE" AFTER MEALS. I.

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Since the original observations by Bence-Jones[1,2] (confirmed some years later by Roberts[3]) showing a temporary decrease in the acidity of urine after meals, some investigators have recorded their inability to find such variations consistently.[4] Although most of the earlier reports on this phenomenon were concerned with acidity as determined by titration, the first[1] of Bence-Jones' communications on the subject was confined to tests made with litmus, and the recent tendency[5,6] to associate the term "alkaline tide," directly or by implication, with variations in the C_H of urine is accordingly a legitimate one, with the historical facts in its favor.

To these two distinct uses of the term "alkaline tide" might be added a third, *viz.* the decrease in "acidity" or increase in "alkalinity" shown by the double titration method recently used by Leathes[7] and others, were it not for the fact that this method does not fulfill the purpose for which it is intended. The method is a determination of the ratio between the amount of alkali required to titrate a given sample of urine to the turning point of phenolphthalein and the amount required to reach the turning point of methyl orange.[8] The results obtained by means of it are supposed to rep-

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[1] Bence-Jones, H., *Phil. Tr. Roy. Soc. London,* 1845, cxxxv, 335.

[2] Bence-Jones, H., *Phil. Tr. Roy. Soc. London,* 1849, cxxxix, 235.

[3] Roberts, W., *Edinburgh Med. J.*, 1859-60, v, 817, 906.

[4] See Hopkins, F. G., and Hope, W. B., *J. Physiol.*, 1898–99, xxiii, 271.

[5] Hasselbalch, K. A., *Biochem. Z.*, 1912, xlvi, 403; 1916, lxxiv, 18.

[6] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1918, xxxiv, 569.

[7] Leathes, J. B., *Brit. Med. J.*, 1919, ii, 165.

[8] A similar procedure had been used earlier by others, but with quite a different object; *viz.*, to determine what proportion of the total amount of weak acids in urine is neutralized (Auerbach, A., and Friedenthal, H., *Arch. Physiol.*, 1903, 397; Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1913, xiv, 81).

resent the ratio of primary to secondary phosphate, and if this were true they could evidently be translated directly into terms of C_H. This claim, however, rests clearly upon the assumption that phosphate is the only weak acid present in urine, and that is well known to be erroneous;[9] nor is the error introduced by this assumption a constant one, and of such a nature as not to invalidate comparative results, for the phosphate content of urine may undergo large and rapid variations without any significant change in the amount of other weak acids.[10]

From this it is clear that ratios thus obtained by the double titration method are not a reliable substitute for C_H determinations, and it is therefore difficult to see any real significance in such ratios, in connection with the problems to which the method has been applied, beyond the information that could be more easily and much more accurately acquired by measuring the C_H directly.

It is interesting to observe the extreme error into which others have fallen in consequence of the idea that urine behaves, in such titrations, as if it were a pure phosphate solution. Collip and Backus[11] have gone a step farther and, assuming that the sum of the two titrations gives "apparently accurate" figures for the phosphate content of urine, have been led to conclude that forced respiration causes a pronounced increase in the excretion of phosphate by the kidneys, although under these conditions the urine is often alkaline and may contain considerable bicarbonate. The figures published by Collip and Backus represent, not phosphate alone, but (approximately) the sum of the phosphate and all other weak acids, including carbonic. Leathes[12] himself (with Broadhurst) has also investigated the same question, and found no rise in phosphate excretion, presumably because he actually determined the phosphate.

As far as I am aware, no one has denied that the C_H of the urine ordinarily diminishes for a time after meals, but the literature on the subject indicates that an unequivocal drop in the titratable acidity under such circumstances is not so general an occurrence. Whether or not this apparent inconsistency is due simply to the comparative paucity of observations on the C_H of urine after meals, it is obvious that titratable acidity and C_H may vary independently, and even in different directions, when any considerable change occurs in the quantity of buffer substances present. Consequently, after a meal rich in phos-

[9] Michaelis has made the same assumption (Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 109).

[10] Fiske, C. H., *J. Biol. Chem.*, 1921, xlix, 171.

[11] Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 568.

[12] Broadhurst, H. C., and Leathes, J. B., *J. Physiol.*, 1920–21, liv, p. xxviii.

phate, the C_H may conceivably fall without any parallel change in titratable acidity, provided always that the urine does not become actually alkaline to the indicator used in the titration. Although acidity titrations have been made in only a few of my experiments under circumstances where this state of affairs is likely to arise, something closely approaching it did occur in one instance (Table I). Here the titratable acidity at 9–10 p.m. (3 hours after the meal) is only a little less than in the period preceding the meal, but the C_H has fallen to about one-tenth of its previous value.

TABLE I.

Experiment 20.

Meal at 7.15 p.m. (for further details see Table II).

Time.	Urine per hour.		
	Volume.	pH	Acidity.
	cc.		*cc. 0.1* N
5– 6 p.m.	60	5.25	11.0
6– 7 "	34	5.15	11.3
7– 8 "	21	5.10	12.3
8– 9 "	31	5.30	13.5
9–10 "	37	6.20	9.0
10–11 "	32	7.25	1.3

The observations reported in this paper (Table II) are taken from experiments (all on the same subject) made primarily for other purposes. They are of interest, in the first place, because most of them show the nature of the variations that may occur in the C_H of urine from hour to hour under the influence of food, whereas longer periods have ordinarily been used by others. Since the changes are often very sudden and transient, they may sometimes completely escape observation when the periods are several hours in duration. (The sharp fall in C_H in the 3rd hour after food in Experiment 37, for example, would have been entirely missed in 2 hour periods.)

Some of these experiments are included to show that with this subject the urine is likely to become alkaline or nearly so at some time after a full meal consisting of meat, with or without other

food, but their main purpose is to point out certain difficulties in deciding whether a similar thing happens after a meal of small proportions.

Concerning the experimental details, beyond what is stated under analytical methods and in the table, nothing need be said except that anything approaching vigorous exercise was always carefully avoided.

Methods.

The hydrogen ion concentration was determined colorimetrically by a modification of the dilution method[13] somewhat different from that ordinarily used.

In each instance, 1 per cent of a 1 hour sample of urine (or its equivalent) was diluted to 10 cc. and compared with a fresh standard (made by adding carbonate-free standard alkali to acetic acid, monopotassium phosphate, or borate-KCl mixture,[14] and diluting to a concentration of 0.01 M). The accuracy of the standards so prepared was frequently controlled by the electrometric method. The indicators used were methyl red, brom-cresol purple, phenol red, and cresol red.[15]

This form of the colorimetric method measures the C_H of the urine, not as secreted, but after dilution to a uniform basis. While this may in some ways be a disadvantage, the results are more useful for certain calculations that have been necessary in investigations to be reported later (*e.g.*, the determination of the ratio of primary to secondary phosphate, and of the total amount of base combined with phosphoric acid), since the high dilution of the solutions diminishes the possibility of complications due to incomplete ionization and the presence of electrolytes.

The acidity was determined by titrating with standard alkali from a micro-burette to match a standard 0.01 M phosphate mixture (pH 7.4), prepared as described above. The indicator was phenol red, and no oxalate was used. The possibility of error from the effect of calcium was, however, eliminated in another way; *viz.*, by repeating each titration in the presence of twice as much water. As it happened, no difference between the two titrations could be detected in the case of any of the urines with which this paper is concerned, but sometimes such differ-

[13] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.

[14] Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

[15] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 63.

ences are encountered, and all that is necessary then is to continue the dilution until it has no further effect. This is satisfactory even with phosphate solutions containing more than enough calcium to combine with all the phosphate.

C_H of Urine after Meals.

The principal facts brought out by the results in Table II are these.[16] The urine usually becomes quite suddenly less acid (and sometimes alkaline) in the 2nd or 3rd hour after a meal. When the meal is a full one (Experiments 14, 15, 20, 36, 38, 44, and 45) the C_H at that time is ordinarily much lower than it is likely to be otherwise. But when the meal is small (Experiments 1, 30, 32, and 37) it is usually impossible to decide whether the "tide" is due directly to the meal under consideration or to a delayed effect of a previous meal, or whether it is in fact anything more than an apparent alkaline tide, representing in reality the recovery from a temporary *increase* in acidity (observed also by Hasselbalch[5]) immediately following the meal.

The reason for this uncertainty may be seen in part from the records for the control experiments (Nos. 20 and 22), which were made following the same breakfast eaten (at about 8 a.m.) in all the others (puffed rice, milk, and coffee), but without further food before 7 p.m. Under these circumstances the pH undergoes minor variations for several hours and does not begin to show any inclination to approach 5.0 progressively until 4 or 5 p.m. The cause of these irregularities doubtless lies in the combined influences of the fixed alkali and potential acid in the food eaten for breakfast, and of the variations in C_H that occur in the morning (and later) when no food is eaten.[5,7,10,12] It may be fairly concluded that the interpretation of similar observations previously published by others is likewise uncertain for the same reasons, and that one cannot be sure of the meaning of any but large variations in the C_H (or titratable acidity) of urine after

[16] Data on the urinary volume are included also (Table III) because of the relation that appears sometimes to exist between volume and acidity. It will be seen, however, that a decrease in C_H in these experiments is accompanied by a drop in volume quite as often as by diuresis.

TABLE II.

pH of Urine.

Breakfast at about 8 a.m. in all experiments (20 gm. puffed rice, 200 cc. milk, and 200 cc. coffee). The figures for the first 6 hours after meals later in the day are in bold-faced type.

Meal in addition to breakfast	250 gm. meat.		120 gm bread, 20 gm. butter, and 200 cc. coffee.				None.	Meat, vegetables, bread and butter, pie, and milk.					
Experiment No.	14	15	1	30	32	37	22	20	38*	36*	44	45	
Meal, p.m.	12.30	12.30	1	12.15	12.30	1		7.15	7 30	7 45	2	2	Time.
Water ingested per hr., cc.	100	100	200	500	None.	100	50	100	100	100	50	100	
Time.													
9–10 a.m.	5.25						5.50	5.50					
10–11 "	5.15	5.50					6.05	6.50					
11–12 m.	5.40	5.75		6.15		6.45	6.45	5.30					
12– 1 p.m.	**5.85**	**6.25**	5.45	**6.00**		6.80	6.30	5.40					
1– 2 "	**7.70**	**7.35**	**5.35**	**5.50**	**5.05**	**6.25**	6.15	5.35					
2– 3 "	**7.45**	**7.10**	**5.45**	**6.20**	**4.95**	**5.55**	6.40	5.45					
3– 4 "	**6.10**	**5.85**	**5.95**	**6.20**	**5.25**	**6.35**	6.50	5.25					
4– 5 "		**5.20**	**6.35**	**6.30**	**5.25**	**5.55**	6.40	5.15			**6.95**	**7.85**	4– 6 p.m.
5– 6 "		**4.90**			**5.40**	**5.75**	6.00	5.25					
6– 7 "						**5.20**	5.65	5.15	5.15		**5.90**	**6.15**	6– 8 p.m.
7– 8 "								**5.10**	**5.00**				
8– 9 "								**5.30**	**5.15**	**5.30**	5.55	5.45	8–10 p.m.
9–10 "								**6.20**	**5.75**				
10–11 "								**7.25**	**6.55**	**7.00**	5.30	5.35	10–12 p.m.
11–12 "									**6.45**				
										5.50	5.60	5.35	12– 2 a.m.

* Lunch (bread, butter, and coffee) at noon.

meals except under circumstances that definitely exclude the influence of food taken earlier.[17]

Whether or not it may be correct to say that the alkaline tide is due to the secretion of hydrochloric acid by the stomach,[18] it is certain that the acidity of urine after meals is influenced by

TABLE III.

Volume of Urine (Cc. per Hour).

Experiment No. / Time.	14	15	1	30	32	37	22	20	38	36	44	45	Time.
9–10 a.m.							87	207					
10–11 "							75	87					
11–12 m.	225	155		570		55	54	83					
12– 1 p.m.	**44**	**47**	78	**383**		61	49	72					
1– 2 "	**71**	**69**	**66**	**325**	**35**	**131**	79	72					
2– 3 "	**66**	**62**	**197**	**496**	**31**	**159**	71	111					
3– 4 "	**56**	**80**	**249**	**275**	**30**	**51**	60	22					
4– 5 "		**167**	**246**	**427**	**30**	**209**	116	25			**84**	**74**	4– 6 p.m.
5– 6 "		**58**			**35**	**186**	34	60					
6– 7 "						**84**	103	34	65		**68**	**116**	6– 8 p.m.
7– 8 "								**21**	**55**				
8– 9 "								**31**	**37**	**40**	114	85	8–10 p.m.
9–10 "								**37**	**37**				
10–11 "								**32**	**38**	**36**	22	36	10–12 p.m.
11–12 "									**34**				
										73	102	49	12– 2 a.m.

[17] The possibility of confusion from such "overlapping" effects was recognized many years ago by Roberts.[3] He also called attention to the necessity of examining the urine at very frequent intervals.

[18] Dodds has recently found that the alveolar CO_2 tension, which normally rises after meals, did not do so in a man whose stomach had been removed, and this is an important point in favor of the gastric secretion theory (Dodds, E. C., *J. Physiol.*, 1920–21, liv, 342; see also Bennett, T. I., and Dodds, E. C., *Brit. J. Exp. Path.*, 1921, ii, 58). The contradictory evidence put forward by Hasselbalch is not at all convincing, being based upon the absence of the alkaline tide after meals in subjects on a carbohydrate-free diet. As a matter of fact, the tide did not disappear in his experiments until the 4th day on this diet, and its absence then can easily be accounted for by the acidosis. It is worth noting that Roberts[3] as long ago as 1860 undoubtedly produced acidosis in his subject by means of a purely "animal" diet, since he also observed that the alkaline tide diminished progressively in intensity in the course of 3 days on such food.

various factors operating at the same time. The maximum alkalinity after a protein meal is often reached at a time when the excretion of sulfate and phosphate (and therefore the rate of production of sulfuric acid, and perhaps of phosphoric acid as well) has reached or is approaching a maximum, and any decrease in acidity occurring then must be in the face of this additional acid production. It is safe enough to suppose that the intensity of the tide (or its appearance at all) will depend not only upon the amount of hydrochloric acid secreted by the stomach (if that is one of the factors involved), but also in an independent way upon the composition of the food. Consequently, even if such a tide is not always evident after meals, that fact alone does not detract from the importance of the phenomenon when it does occur.

INORGANIC PHOSPHATE AND ACID EXCRETION IN THE POSTABSORPTIVE PERIOD.

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The introduction, in the middle of the 19th century, of Liebig's titration method for the determination of phosphate in urine stimulated the first investigations on the subject of diurnal variations in phosphate excretion.

As part of a comprehensive program instituted in connection with the *Verein für gemeinschaftliche Arbeiten zur Förderung der wissenschaftlichen Heilkunde*, two students (Winter[1] and Mosler[2]), working under the direction of Vogel, observed that the rate of phosphate excretion in men on ordinary diets reaches its lowest point in the morning. Their experimental days were divided into 3 or 4 periods of varying length, and the result of this was that the time of occurrence of the minimum was not well defined. Shortly afterwards these observations were repeated by Beneke[3] with uniform 3 hour periods (except at night) and he found the lowest phosphate output (averaging 24 mg. of phosphorus per hour) to occur between 7 and 10 a.m., with 39, 54, 52, and 45 mg. per hour in the succeeding 3 hour periods, and 39 mg. per hour during the night.[4]

Some years later, von Haxthausen[6] arrived at a similar conclusion, the uranium titration method having meanwhile been devised, and many others since have called attention to the low rate of phosphate excretion in the morning after the first meal of the day (Zuelzer,[7] Edlefsen,[8,9] Speck,[10]

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[1] Winter, A., Dissertation, Giessen; cited by Beneke, F. W., *Arch. wissensch. Heilkunde*, 1854, i, 667.

[2] Mosler, F., Dissertation, Giessen; cited by Beneke, F. W., *Arch. wissensch. Heilkunde*, 1854, i, 670.

[3] Beneke, F. W., *Arch. wissensch. Heilkunde*, 1854, i, 391, 571.

[4] de Jager,[5] in reporting Beneke's figures, has retained an error in the original in the average for the second period.

[5] de Jager, L., *Z. physiol. Chem.*, 1898, xxiv, 303.

[6] von Haxthausen, H. A., Inaugural dissertation, Halle, 1860; cited by Edlefsen.[8]

[7] Zuelzer, W., *Virchows Arch. path. Anat.*, 1876, lxvi, 223, 282.

[8] Edlefsen, G., *Cent. med. Wissensch.*, 1878, xvi, 513.

[9] Edlefsen, G., *Deutsch. Arch. klin. Med.*, 1881, xxix, 409.

[10] Speck, *Arch. exp. Path. u. Pharmakol.*, 1881–82, xv, 81.

Ott,[11] Roeske,[12] de Jager,[5] Sherman and Hawk,[13] Hawk,[14] Hawk and Chamberlain,[15] Cathcart, Kennaway, and Leathes,[16] and some others with less definite results). The observation has been extended also to dogs receiving but one meal in the course of 24 hours (Feder,[17] Vogt,[18] Loeb,[19] Wolf and Österberg[20]).

The earliest investigators of this subject were engaged in breaking ground in a field that had not previously been touched. They could hardly, at first, have been in a position to plan their experiments in such a way as to avoid misinterpretations due to the influence of food. Their observations on the curve of phosphate excretion accordingly occasioned no surprise, for their results with sulfate were essentially the same, and that was just what they expected on the basis of the erroneous conception then prevailing concerning the distribution of phosphorus in proteins.[21] They were naturally, therefore, inclined to ascribe their findings chiefly to the gradual fall in phosphate excretion during the night (inasmuch as that was the longest period without food), and slow absorption was to them a sufficient explanation for the failure of the phosphate curve to rise immediately after the first meal. No one since, in fact, has seriously questioned this way of accounting for the facts, with the exception of Loeb[19], and his speculations on the subject were not supported by experimental evidence of any sort.

My attention was directed some time ago to the matter of variations in inorganic phosphate excretion during the 1st day

[11] Ott, A., *Z. physiol. Chem.*, 1886, x, 1.

[12] Roeske, G., Dissertation, Greifswald, 1897; cited by Sherman and Hawk.[13]

[13] Sherman, H. C., and Hawk, P. B., *Am. J. Physiol.*, 1900–01, iv, 25.

[14] Hawk, P. B., *Am. J. Physiol.*, 1903–04, x, 115.

[15] Hawk, P. B., and Chamberlain, J. S., *Am. J. Physiol.*, 1903–04, x, 269.

[16] Cathcart, E. P., Kennaway, E. L., and Leathes, J. B., *Quart. J. Med.*, 1907–08, i, 416.

[17] Feder, L., *Z. Biol.*, 1881, xvii, 531.

[18] Vogt, H., *Beitr. chem. Physiol. u. Path.*, 1906, viii, 409.

[19] Loeb, A., *Z. Biol.*, 1910–11, lv, 167.

[20] Wolf, C. G. L., and Österberg, E., *Biochem. Z.*, 1912, xli, 111.

[21] This, together with a general lack of knowledge about the nature of the phosphorus compounds in foods (particularly meat), undoubtedly influenced the interpretation of many later investigations in the field of phosphate metabolism, including the researches on the nitrogen-phosphorus ratio initiated by Bischoff (Bischoff, E., *Z. Biol.*, 1867, iii, 309). The term "phosphoprotein" (or its equivalent, "nucleoalbumin") is even now too often loosely used, although Plimmer and Scott have failed to find evidence for the existence of any such substance in animal tissues, with the possible exception of the pancreas (Plimmer, R. H. A., and Scott, F. H., *J. Chem. Soc.*, 1908, xciii, 1699).

of fasting by observations on 1 hour urines collected during the morning. These revealed a surprising and apparently unaccountable increase in the phosphate output, reaching its maximum at about the middle of the afternoon (see Experiment 57, Table I).

TABLE I.

Fasting since 7 p.m. of Preceding Day.

Experiment 57. 100 cc. of water per hour.

Time.	Urine (per hour).	
	Volume.	Inorganic P.
	cc.	*mg.*
7– 8 a.m.	36	24.7
8– 9 "	25	22.0
9–10 "	22	12.5
10–11 "	75	12.6
11–12 m.	136	13.5
12– 1 p.m.	85	15.4
1– 2 "	40	18.4
2– 3 "	95	25.0
3– 4 "	63	25.8
4– 5 "	178	26.6
5– 6 "	114	24.7
6– 7 "	38	23.7
7– 8 "	34	22.5
8– 9 "	26	22.3
9–10 "	31	22.5

Time.	Urine (per hour).									
	Volume.	pH	Inorganic P.	NH_3-N	Inorganic sulfate S.	Phosphoric acid.	Ammonia.	Sulfuric acid.	Acidity.	Acidity + NH_3.
	cc.		*mg.*	*mg.*	*mg.*	*cc. 0.1 M*	*cc. 0.1 N*	*cc. 0.1 N*	*cc. 0.1 N*	*cc. 0.1 N*
8–10 a.m.	24	5.70	17.3	31.0	16.4	5.6	22.1	10.2	7.2	29.3
10–12 m.	106	6.00	13.1	31.3	14.4	4.2	22.3	9.0	5.9	28.2
12– 2 p.m.	63	5.80	16.9	27.2	11.9	5.4	19.4	7.4	7.0	26.4
2– 4 "	79	5.75	25.4	26.2	12.0	8.2	18.7	7.5	8.9	27.6
4– 6 "	146	5.75	25.7	22.4	14.7	8.3	16.0	9.2	10.0	26.0
6– 8 "	36	5.50	23.1	21.9	13.3	7.4	15.6	8.3	9.9	25.5
8–10 "	29	5.55	22.4	20.0	13.8	7.2	14.3	8.6	9.4	23.7

Further inquiry immediately showed that this rise begins

soon after the drop long known to occur during the night has reached its lowest point, which may be as low as 6.3 mg. of phosphorus per hour under these conditions.[22] A curve of this same general form has now been found, without exception, in about 40 such experiments on four subjects, and some of the observations on three of these will be published later in another connection. The present paper will be confined to experiments selected from some 25 performed on the fourth subject, involving determinations, not only of phosphate, but also of several other factors concerned in the question of acid excretion (which likewise is subject to variations of considerable magnitude during the post-absorptive period), the object being to attempt to learn the reasons for these changes as a whole. No vigorous exercise was taken during the course of any of these experiments.

It will readily be seen that the situation just described,[23] occurring when the rising part of the phosphate curve cannot be accounted for by food recently eaten, bears no necessary relation to the similar course of events that many have observed (at various times in the last 70 years) following breakfast, inasmuch as one could not have been predicted from the other. Nevertheless, it is not at all impossible that the explanation of both, insofar as they are qualitatively alike, will ultimately prove to be the same.

One other matter respecting the phosphate curves themselves

[22] A preliminary report on this point, made at the meeting of the American Society of Biological Chemists in December, 1919, was published in March, 1920 (Fiske, C. H., *J. Biol. Chem.*, 1920, xli, p. lix). Similar observations have since been recorded by Broadhurst and Leathes in a paper presented before the Physiological Society in the following July, and published in December (Broadhurst, H. C., and Leathes, J. B., *J. Physiol.*, 1920–21, liv, p. xxviii).

[23] Feder[17] published two fasting experiments (on dogs) intended to serve as controls for his meat feeding experiments. Provided that certain assumptions are made about the time of day represented by his periods, the results of these experiments give a curve not unlike those reported in this paper. Feder, however, dismissed them as accidental variations. Edlefsen[9] likewise found in one experiment a rise in phosphate excretion between 12 m. and 6 p.m. of the 2nd day of fasting in man, with lower figures for the 6 hour periods preceding and following. This is undoubtedly the same phenomenon that I have described, but the 1st fast day of Edlefsen's experiment did not show it, nor would mine if 6 hour periods had been used.

requires to be mentioned. In all those experiments in which the level of inorganic phosphorus excretion, in its rise during the late morning and early afternoon, eventually exceeded 25 mg. per hour, there followed (whenever the experiment was continued for a few hours) a further slight fall to between 20 and 25 mg. In the single experiment that was continued later than 6 p.m., the curve finally became horizontal for some hours (Table I), and essentially the same thing happened in an experiment on another subject (not now included), which lasted until midnight.

These oscillations in the curve of phosphate excretion (which an inspection of the tables will show to be entirely unconnected with the volume of urine), naturally suggested some relation to the alkaline tide observed by Hasselbalch[24] during the morning in subjects who had been without food since the previous noon, and an investigation was accordingly begun, involving simultaneous observations on both these things. The subject of these experiments ate, throughout, an ordinary mixed diet divided into the customary three meals a day, with no attempt to maintain constancy of composition, since this was readily shown to be unnecessary for the purpose. It was shortly found that, in this subject under such circumstances, no very marked alkaline tide occurred during the morning, whether the last meal had been taken on the evening before or at about the middle of the preceding day (Tables I and VI). In order to intensify this tide, with the hope of helping to unravel its relation to the phosphate curve, the device was adopted of drinking varying quantities of milk at about midnight of the day before. The temporary drop in the C_H of the urine during the morning was then ordinarily much more pronounced, and the more so the larger the amount of milk taken (Tables II and III). The effect was similar when the food eaten at that time included other things besides milk (Table IV).

The variations observed in the excretion of inorganic sulfate can furnish no basis for explaining the alkaline tide by alterations in the rate of sulfuric acid production, since the tendency throughout is for the inorganic sulfate content of the urine to decrease gradually to an approximately constant level, with no significant rise in the latter part of the day, and since even the decrease is

[24] Hasselbalch, K. A., *Biochem. Z.*, 1912, xlvi, 403; 1916, lxxiv, 18.

TABLE II.

200 Cc. of Milk at Midnight.

Time.	Urine (per hour).		
	Volume.	pH	Inorganic P.
Experiment 31. Fasting. 200 cc. of water every 30 minutes.			
	cc.		mg.
8– 9 a.m.	40	5.00	22.2
9–10 "	215	5.85	12.2
10–11 "	474	6.80	6.3
11–12 m.	397	6.60	7.6
12– 1 p.m.	421	6.40	12.5
1– 2 "	358	6.05	18.1
2– 3 "	358	6.05	18.1
3– 4 "	330	5.85	25.7
4– 5 "	300	5.50	27.8
Experiment 35. Fasting. 100 cc. of water per hour.			
6– 7 a.m.	22	5.05	32.2
7– 8 "	29	5.10	24.4
8– 9 "	95	5.25	18.9
9–10 "	67	6.05	13.6
10–11 "	130	7.10	14.6
11–12 m.	166	7.15	16.7

Experiment 25. Fasting. 100 cc. of water per hour.

Time.	Urine (per hour).							
	Volume.	pH	Inorganic P.	NH_3-N	Inorganic sulfate S.	Phosphoric acid.	Ammonia.	Sulfuric acid.
	cc.		mg.	mg.	mg.	cc. 0.1 M	cc. 0.1 N	cc. 0.1 N
8– 9 a.m.	34	5.20	24.7	29.2	24.4	8.0	20.8	15.2
9–10 "	36	5.15	13.2	24.7	21.9	4.3	17.6	13.6
10–11 "	67	5.30	11.2	23.7	21.6	3.6	16.9	13.5
11–12 m.	65	5.35	12.3	21.6	18.2	4.0	15.4	11.3
12– 1 p.m.	46	5.20	15.2	24.0	16.5	4.9	17.2	10.3
1– 2 "	34	5.10	19.1	22.8	16.9	6.2	16.3	10.6
2– 3 "	52	5.15	23.8	25.8	16.3	7.7	18.4	10.2
3– 4 "	57	5.10	28.5	24.5	17.3	9.2	17.5	10.8
4– 5 "	44	5.00	28.7	20.7	16.9	9.2	14.8	10.6
5– 6 "	25	4.90	28.5	19.7	16.1	9.2	14.1	10.1

TABLE II—*Concluded.*

Time.	Urine (per hour).									
	Volume.	pH	Inorganic P.	NH_3-N	Inorganic sulfate S.	Phosphoric acid.	Ammonia.	Sulfuric acid.	Acidity.	Acidity + NH_3.
Experiment 50. Fasting. 200 cc. of water per hour.										
	cc.		*mg.*	*mg.*	*mg.*	*cc. 0.1* M	*cc. 0.1* N	*cc. 0.1* N	*cc. 0.1* N	*cc. 0.1* N
7– 8 a.m.	37	5.40	27.8	32.1	28.1	8.9	22.9	17.6	11.8	34.7
8– 9 "	35	5.45	20.3	29.5	26.1	6.5	21.1	16.3	9.1	30.2
9–10 "	75	5.45	15.8	29.3	23.4	5.1	20.9	14.6	8.7	29.6
10–11 "	204	5.75	12.5	28.0	21.3	4.0	20.0	13.3	7.7	27.7
11–12 m.	115	5.75	14.2	20.0	19.5	4.6	14.3	12.2	7.1	21.4
12– 1 p.m.	282	5.95	16.5	19.6	18.8	5.3	14.0	11.7	7.5	21.5
1– 2 "	193	5.85	17.2	22.3	18.1	5.5	15.9	11.3	7.9	23.8
2– 3 "	152	5.65	23.0	23.1	17.6	7.4	16.5	11.0	9.9	26.4
3– 4 "	153	5.55	28.2	24.6	19.2	9.1	17.6	12.0	11.8	29.4

accompanied by a parallel change at least as great in the ammonia excretion.[25] A certain correspondence is evident between the phosphate and the C_H, although the latter sometimes continues to fall after the phosphate has begun to rise again. The parallelism between phosphate and titratable acidity is much closer and fails at only one point, *viz.* the drop in acidity at the trough of the wave is likely to be more pronounced than the associated change in phosphate, and this corresponds with the occurrence of a fall in C_H, which is most marked at about that point.

To judge from present tendencies in discussions of neutrality regulation in the animal body, many would be inclined to believe

[25] Organic acid determinations by the method of Van Slyke and Palmer (Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, 1920, xli, 567) in several of these experiments yielded results for the various urines of any one experiment that were hardly distinguishable from one another. They were of the same order of magnitude, when reduced to the same basis, as those reported by the originators of the method for 24 hour urines, and since they cannot be regarded as anything but approximations the figures are omitted. They have served, however, to eliminate organic acids as a factor in the present problem.

that the phosphate output in these experiments falls because there is less acid requiring to be neutralized, and rises again later because of increased acid production; in other words, that the amount of phosphate excreted is determined by the amount of other acids calling for removal from the body. For such an

TABLE III.

500 Cc. of Milk at Midnight.

Experiment 21. Fasting. 50 cc. of water per hour.

Time.	Urine (per hour).		
	Volume.	pH	Inorganic P.
	cc.		*mg.*
6– 7 a.m.	24	5.50	35.9
7– 8 "	38	5.35	35.0
8– 9 "	55	5.40	29.9
9–10 "	45	5.35	24.6
10–11 "	57	6.50	17.2
11–12 m.	170	6.85	19.4
12– 1 p.m.	73	7.05	32.7

Experiment 65. Fasting. 200 cc. of water every 2 hours.

Time.	Urine (per hour).									
	Volume.	pH	Inorganic P.	NH_3-N	Inorganic sulfate S.	Phosphoric acid.	Ammonia.	Sulfuric acid.	Acidity.	Acidity + NH_3.
	cc.		*mg.*	*mg.*	*mg.*	*cc. 0.1* M	*cc. 0.1* N	*cc. 0.1* N	*cc. 0.1* N	*cc. 0.1* N
6– 8 a.m.	65	5.50	40.0	23.5	23.1	12.9	16.8	14.4	11.8	28.6
8–10 "	26	5.55	27.6	20.0	20.2	8.9	14.3	12.6	10.0	24.3
10–12 m.	55	7.25	18.3	8.7	18.4	5.9	6.2	11.5	1.4	7.6
12– 2 p.m.	141	6.95	21.0	6.6	15.2	6.8	4.7	9.5	2.8	7.5
2– 4 "	78	6.50	33.1	8.4	14.9	10.7	6.0	9.3	6.6	12.6

interpretation there is, as far as I know, no evidence whatever, and it is easily shown (Table V) that the general form of the curve of phosphate excretion in the postabsorptive period is not at all altered by the administration of sodium bicarbonate, whether this is given some hours before the beginning of the experiment (Experiment 40) or at a time when the curve has begun to rise (Experiment 39).

TABLE IV.

Meal at Midnight.

Experiment 9. Fasting. At 12 m. (preceding), 1 egg, bread and butter, apple pie, and 500 cc. of milk. 100 cc. of water per hour.

Time.	Urine (per hour).									
	Volume.	pH	Inorganic P.	NH_3-N	Inorganic sulfate S.	Phosphoric acid.	Ammonia.	Sulfuric acid.	Acidity.	Acidity + NH_3.
	cc.		*mg.*	*mg.*	*mg.*	*cc. 0.1* M	*cc. 0.1* N	*cc. 0.1* N	*cc. 0.1* N	*cc. 0.1* N
7– 8 a.m.	36	5.30	47.6	35.9	35.7	15.3	25.6	22.3	16.0	41.6
8– 9 "	39	5.20	37.8	31.5	27.6	12.2	22.5	17.3	12.2	34.7
9–10 "	39	5.20	37.8	31.5	27.6	12.2	22.5	17.3	12.2	34.7
10–11 "	74	6.20	21.2	17.2	26.7	6.8	12.3	16.7	6.0	18.3
11–12 m.	257	6.60	20.6	16.0	23.0	6.7	11.4	14.4	4.8	16.2
12– 1 p.m.	186	6.75	19.9	11.1	19.2	6.4	7.9	12.0	3.4	11.3
1– 2 "	100	6.75	23.8	11.3	19.7	7.7	8.1	12.3	3.5	11.6
2– 3 "	239	6.60	29.8	10.5	15.8	9.6	7.5	9.9	5.4	12.9
3– 4 "	170	6.35	31.4	10.1	14.4	10.1	7.2	9.0	6.0	13.2
4– 5 "	130	6.10	30.3	14.4	16.6	9.8	10.3	10.4	8.1	18.4
5– 6 "	52	5.30	25.0	20.2	14.7	8.1	14.4	9.2	9.0	23.4

TABLE V.

Effect of $NaHCO_3$ on Phosphate Excretion.

Time.	Urine (per hour).			
	Volume.	pH	Inorganic P.	
Experiment 39. Fasting. 200 cc. of milk at midnight. 100 cc. of water per hour.				
	cc.		*mg.*	
11–12 m.	49	7.25	7.2	
12– 1 p.m.	117	6.95	14.8	
1– 2 p.m.	70	7.75	18.7	1 p.m. 10 gm. of $NaHCO_3$ *per os.*
2– 3 "	46	8.35	25.3	
Experiment 40. Fasting. 200 cc. of milk at midnight. 5 gm. of $NaHCO_3$ in 200 cc. of water at 4 a.m. 100 cc. of water per hour.				
9–10 a.m.	72	7.65	15.2	
10–11 "	55	7.65	11.7	
11–12 m.	94	7.60	13.9	
12– 1 p.m.	87	7.50	21.9	

The observations as a whole, insofar as they can be accounted for at all by those factors that have been determined in these experiments, can be interpreted only upon the basis of a decrease (followed by a rise) in the rate of production of phosphoric acid during the morning, or, what is perhaps more probable, although its effect on the composition of the urine might well be the same, by an active retention of phosphate (phosphoric acid or primary phosphate), which later in the day is "released."

TABLE VI.

Phosphate Excretion between Midnight and Noon.

Experiment 44. Fasting since 2 p.m. 100 cc. of water every 2 hours.

Time.	Urine (per hour).		
	Volume.	pH	Inorganic P.
	cc.		*mg.*
12– 2 a.m.	102	5.60	33.0
2– 4 "	123	5.65	31.0
4– 6 "	33	5.45	28.4
6– 8 "	32	5.60	18.7
8–10 "	71	5.80	17.1
10–12 m.	52	5.50	15.6

One experiment (Table VI) must be referred to again because of its bearing on the possible validity of this interpretation. In this experiment, which continued from midnight until the next noon, no food having been taken since 2 p.m., it will be seen that the phosphate output underwent only a gradual drop between midnight and 6 a.m., but then fell suddenly in the next 2 hours. If the falling curve as a whole were to be accounted for solely on the basis of the gradual excretion of phosphate derived from the last meal, it would be difficult to explain this marked irregularity.

The explanation (phosphate retention) that I have offered as the one most probable from the facts available is not established by the experiments now reported, nor do I believe that it will *by itself* completely account for the situation. The problem is now being investigated in several directions with the hope of throwing more light upon it.

EXPERIMENTAL.

Most of the figures given for inorganic phosphate were obtained with the colorimetric method of Bell and Doisy,[26] which has proved to be quite accurate enough for the purpose at hand.[27] The analyses in Experiment 25 and those preceding it in the series were made by the titration method recently described.[27] and this has been used also in many isolated instances as a check on the colorimetric method. The results have served to show[27] that the latter is entirely satisfactory, when an accuracy of about 2 per cent is sufficient, under all the conditions met in these experiments, whether the phosphate excretion is high or low.

Inorganic sulfate was determined by precipitation with benzidine after removing the phosphate;[28] ammonia by aeration (into 5 cc. of 0.02 N HCl diluted with water) followed by titration (methyl red); C_H and acidity in the manner described in the preceding paper.[29]

The urines, immediately after collecting, were diluted to some convenient volume (most often 100 cc.) and the analyses begun immediately, except with samples obtained late in the day, which were preserved over night with chloroform in the cold room. The sulfate determinations were sometimes all postponed until the following day, but the phosphate was then removed (with magnesium carbonate, etc.) while the urines were fresh; the alkaline filtrates resulting keep for some time.

[26] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.
[27] Fiske, C. H., *J. Biol. Chem.*, 1921, xlvi, 285.
[28] Fiske, C. H., *J. Biol. Chem.*, 1921, xlvii, 59.
[29] Fiske, C. H., *J. Biol. Chem.*, 1921, xlix, 163

A BUFFER SOLUTION FOR COLORIMETRIC COMPARISON.

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In the use of standard buffer solutions for colorimetric comparison where more than a restricted range of reaction is required, it has been necessary in the past to make use of several solutions. The author has developed a system requiring but two stock solutions and covering a range of from pH 2.2 to pH 8.0 which approximately includes the limits of reaction for arable soils and physiological media. The materials used are as follows: 0.2 M disodium phosphate[1] and 0.1 M citric acid, combined in such volumes as to make 20 cc. of the mixture.

The disodium phosphate employed was recrystallized three times. A 0.2 M solution was prepared by titration against HCl, using methyl orange as indicator. Although the titration end-point was not distinct, the error in the resulting mixtures was usually not appreciable, seldom exceeding 0.01 pH. On account of the variable water of crystallization content of the phosphate salt[2] the stock solution was standardized by titration in order to have a reproducible method. Upon exposure to the air for a period of 2 weeks the water of crystallization of disodium phosphate is reduced to 2 molecules. If, after exposure, a quantity of the salt is kept on hand in a closed container and the correct weight of the salt required to produce the proper concentration of stock solution determined by titration, it is possible at any time to make up the stock solution by simply weighing out the salt. The citric acid was recrystallized at least twice before using.

[1] Molds may develop in phosphate solutions under suitable conditions, but this source of trouble has been obviated by Martin[2] by shaking the solutions with a little calomel for a few minutes and then filtering.

[2] Martin, C. J., *Biochem. J.*, 1920, xiv, 98.

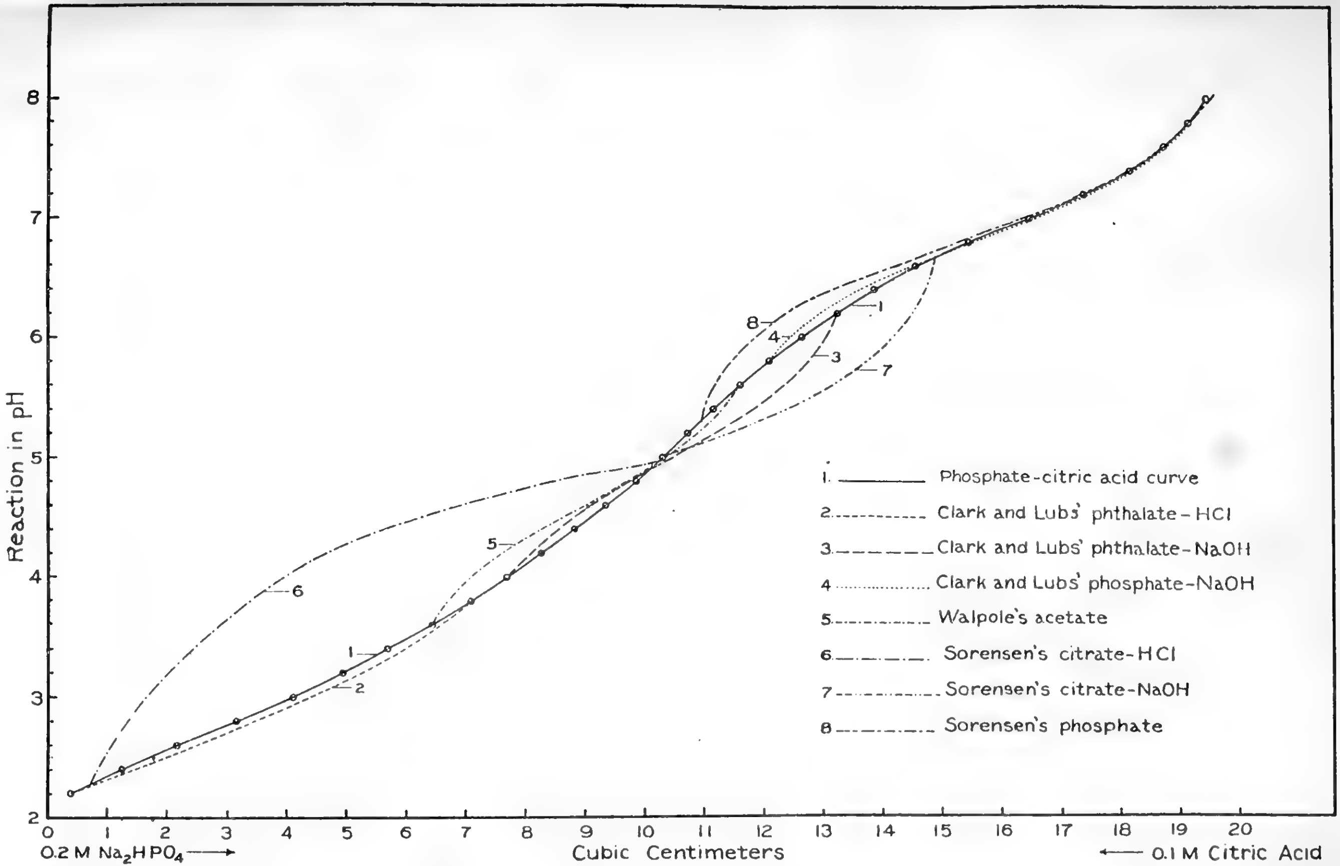
The 0.1 M stock solution was standardized by titration against NaOH solution which had been prepared with boiled water and protected from CO_2. Barium hydroxide was also used as recom-

TABLE I.

pH required.	0.2 M Na_2HPO_4.	0.1 M citric acid.
	cc.	cc.
2.2	0.40	19.60
2.4	1.24	18.76
2.6	2.18	17.82
2.8	3.17	16.83
3.0	4.11	15.89
3.2	4.94	15.06
3.4	5.70	14.30
3.6	6.44	13.56
3.8	7.10	12.90
4.0	7.71	12.29
4.2	8.28	11.72
4.4	8.82	11.18
4.6	9.35	10.65
4.8	9.86	10.14
5.0	10.30	9.70
5.2	10.72	9.28
5.4	11.15	8.85
5.6	11.60	8.40
5.8	12.09	7.91
6.0	12.63	7.37
6.2	13.22	6.78
6.4	13.85	6.15
6.6	14.55	5.45
6.8	15.45	4.55
7.0	16.47	3.53
7.2	17.39	2.61
7.4	18.17	1.83
7.6	18.73	1.27
7.8	19.15	0.85
8.0	19.45	0.55

mended by Sorensen and given by Clark,[3] but sodium hydroxide was found to be as accurate and more convenient. The correct weight of citric acid required to make the stock solution can also be determined by titration.

[3] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.


8
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Reaction in pH
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0.2 M Na2HPO4 ⟶
Cubic Centimeters
⟵ 0.1 M Citric Acid
1. Phosphate-citric acid curve
2. Clark and Lubs' phthalate-HCl
3. Clark and Lubs' phthalate-NaOH
4. Clark and Lubs' phosphate-NaOH
5. Walpole's acetate
6. Sorensen's citrate-HCl
7. Sorensen's citrate-NaOH
8. Sorensen's phosphate

FIG. 1.

The pH values of the mixtures were determined electrometrically by use of the chain; Hg | HgCl | NKCL | saturated solution of KCl | H_2 | Pt. No allowance was made for liquid potential. No attempt was made to maintain a constant temperature. However, the temperature of both calomel electrode and buffer solution were taken into account in computing the pH values. Clark's[3] extension of Sorensen's values for the normal calomel electrode was used with the necessary interpolations. Three extra calomel electrodes were employed for checking the accuracy of the one in general use. A Leeds and Northrup type K potentiometer and type R sensitive galvanometer were used for making the electrometric measurements. The electrode was of the platinum wire variety. The hydrogen was generated electrolytically and passed first through an acid permanganate solution, next through a hot tube, and finally through a wash bottle containing distilled water.

A graph was constructed in which the pH values (determined electrometrically) of various mixtures of phosphate and citric acid solution, so arranged that the total volume was in all cases 20 cc., were plotted against the volumes of the two solutions. By interpolation, using the curve so obtained, it was possible to arrive at the proper volumes of the two solutions which when mixed would give 20 cc. of a solution having any desired reaction. The values given in Table I were obtained in this way and checked by actually preparing the solutions and measuring the pH values by the electrometric method. In all cases the variation of the observed from the calculated pH was 0.01 or less.

Fig. 1 gives the titration curve for the foregoing system of buffers. For comparison, there is also given the titration curves of well known standard buffer solutions.[3]

The shape of the phosphate-citric acid curve of Fig. 1 indicates that the mixtures are well suited for colorimetric determinations of pH.

THE CALCIUM CONTENT OF BLOOD PLASMA AND CORPUSCLES IN THE NEW-BORN.*

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(Received for publication, August 28, 1921.)

In a recent communication by Jones and Nye (1) the distribution of calcium and phosphoric acid in the blood of normal children was reported. As found in that series of observations the average calcium content of the blood of normal children ranging in age from 4 weeks to 14 years was as follows: whole blood, 9.4 mg. per 100 cc.; corpuscles, 8.7 mg.; and plasma, 10.0 mg. So far as we are aware, no data have been published on the calcium content of plasma and corpuscles in the blood of the new-born. The observations herein reported on the calcium content of whole blood, corpuscles, and plasma in infants ranging in age from 4 hours to 12 days are a continuation of the calcium-phosphorus studies previously reported from this department.

Technique.

Lyman's (2) nephelometric method was used, the technique described by Jones and Nye being employed except in a few minor details. In previous work in this laboratory the plan of checking our technique by making determinations on whole blood, corpuscles, and plasma and comparing the actual whole blood value with that found by calculation from its component parts was adopted and followed in this series of observations. The blood was taken by means of syringe from the superior longitudinal sinus about 4 hours after feeding. Approximately 15 cc. were collected, 1 drop of a saturated solution of sodium citrate to 5 cc. of blood being used to prevent coagulation. As a precaution against

* Part of the expense of this investigation was borne by a grant from the William H. Crocker fund for research in pediatrics.

an exchange of ions between the corpuscles and plasma, the portion of blood to be used for these determinations (10 cc.) was introduced quickly through a short piece of glass tubing into a graduated tube containing 2 drops of the citrate solution and 1 cc. of paraffin oil was stirred gently, and centrifugated immediately. When more

TABLE I.

Calcium Content of the Blood of Normal Infants from Birth to 12 Days of Age.

Sex.	Age.	No. of determination included in average.	Average hematocrit reading.	Average calcium values per 100 cc.		
				Whole blood.	Corpuscles.	Plasma.
	days		*per cent*	*mg.*	*mg.*	*mg.*
Boys.	0–2	5	55.1	8.7	5.8	12.1
	2–4	6	50.5	8.9	5.4	12.3
	4–6	6	49.1	8.8	5.4	12.3
	6–8	6	45.7	9.0	5.2	12.3
	8–10	6	47.2	8.9	5.1	12.3
	10–12	6	40.8	9.4	5.0	12.[illegible]
Average...........	0–12	35	48.6	9.0	5.3	12.3
Girls.	0–2	6	54.8	8.2	4.9	12.2
	2–4		49.2	8.5	4.9	12.0
	4–6	5	49.0	8.6	4.7	12.2
	6–8	5	45.7	8.8	4.7	12.3
	8–10	6	43.9	8.9	4.5	12.1
	10–12	5	42.8	9.1	4.7	12.5
Average...........	0–12	33	47.4	8.7	4.7	12.2
Boys and girls.	0–2	11	55.0	8.4	5.4	12.2
	2–4	12	49.9	8.7	5.2	12.2
	4–6	11	49.1	8.7	5.1	12.3
	6–8	11	45.7	8.9	5.0	12.3
	8–10	12	45.5	8.9	4.8	12.3
	10–12	11	41.9	9.3	4.9	12.4
Average...........	0–12	68	48.0	8.8	5.0	12.3

than one determination was made on an individual, the same centrifuge tube was used each time and the hematocrit reading carefully taken, due allowance being made for the amount of citrate solution which was added. Each sample of blood was analyzed immediately after collection.

After a few analyses according to the procedure described by Jones and Nye the calcium content of the corpuscles was found to be so low that it was necessary to change the dilution in order

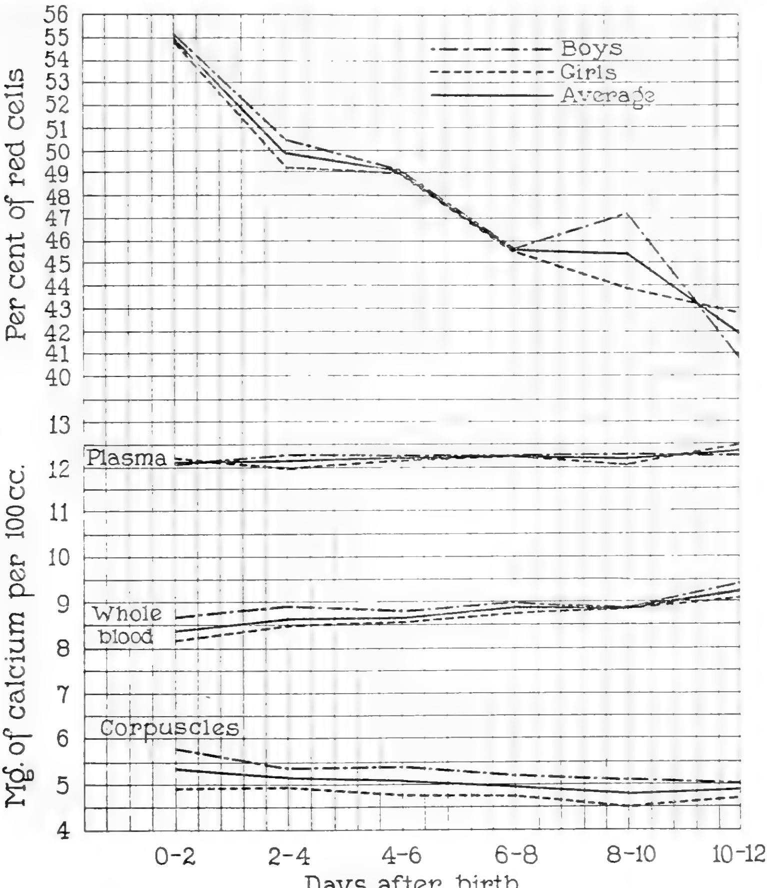

FIG. 1. Graphic representation of the calcium content of the blood of normal infants from birth to 12 days of age.

to obtain satisfactory nephelometric readings with the standard used. 3 cc. of corpuscles were laked with an equal volume of distilled water instead of 9 cc. and 5 cc. of the 1:1 dilution were added to 20 cc. of the trichloroacetic acid solution. Determina-

tions were made on washed and unwashed corpuscles, and since the differences between the values obtained were insignificant, washing with 0.9 per cent NaCl was discontinued. 68 determinations were made on 22 infants, 12 boys and 10 girls. In 12 cases the first sample of blood was taken as soon after birth as possible, within 12 hours, and at intervals of 3 or 4 days thereafter until four analyses had been made. In 7 cases, the first sample was taken during the 2 to 4 day period, and in the remainder (3 cases) on the 9th day.

TABLE II.

Averages and Variations in the Calcium Content of the Blood of Normal Infants Ranging in Age from 4 Hours to 12 Days, and of Normal Children from 4 Weeks to 14 Years.

		Calcium per 100 cc.			Red blood cells.
		Whole blood.	Corpuscles.	Plasma.	
		mg.	*mg.*	*mg.*	*per cent*
Low	Infants........	8.1	4.2	11.4	37.1
	Children.......	5.6	5.2	5.5	30.1
Average	Infants........	8.8	5.0	12.3	48.0
	Children.......	9.4	8.7	10.0	38.2
High	Infants........	10.3	6.9	13.2	64.5
	Children.......	12.4	12.0	12.4	44.0

The results of the analyses are given in Table I and Fig. 1.

For convenience in comparison, averages and variations in the calcium content of the blood of infants and older children are given in Table II.

DISCUSSION.

An examination of Tables I and II shows that the average calcium content of blood plasma is higher in the new-born than in older children. This is in agreement with the findings of Meigs, Blatherwick, and Cary (3) who showed that in heifers the calcium content of plasma is highest at birth and tends to become lower with advancing age up to 6 months. The value for whole blood (8.8 mg. per 100 cc.) is slightly less than that (9.5 mg.) reported by Brown, MacLachlan, and Simpson (4) in normal

infants under 1 year of age, while the average content of corpuscles (5.0 mg.) is markedly less than that (8.7 mg.) found by Jones and Nye in older children. It is interesting to note (Table I) the constancy of the plasma values throughout the series, the average for each period varying less than 0.4 mg. per 100 cc. from the general average. Apparently in man the drop in the plasma content does not occur during the first 12 days of life. In general, the corpuscle values tend to decrease slightly during the first few days, the difference, however, in the majority of cases was well within the limits of experimental error of the method and much emphasis should not be laid upon it. On the other hand, there is a tendency for the whole blood values to increase. This is what one would expect with relatively constant values for plasma and corpuscles and a marked decrease in the percentage of cells. As Jones and Nye found in older children, the calcium values in the new-born tend to run higher in boys than in girls. The significance of this difference is not understood.

Probably the most interesting feature of the hematocrit curves is their step-like character. This is shown most strikingly in the general average. The sudden rise in the boys' curve in the fifth period can be accounted for by the very high percentage of cells in one infant, from whom the first sample of blood was taken on the 9th day. A few preliminary observations on the fate of the red blood cells during the first few days of life indicate that the decrease in the percentage of corpuscles is due to a relative increase in plasma volume rather than to a destruction of the cells themselves. Work on this subject is now in progress.

SUMMARY.

A series of observations on the calcium content of the blood of normal infants ranging in age from 4 hours to 12 days is reported. 68 determinations on 22 infants, 12 boys and 10 girls, were made, the average values being as follows: whole blood, 8.8 mg. per 100 cc.; corpuscles, 5.0 mg.; and plasma, 12.3 mg. The average for plasma is higher than that reported in older children while corpuscle and whole blood values are less.

The 12 days included in the series of observations were divided into six periods of 2 days each, and the results of the analyses made during each period averaged and plotted. The plasma values

remained constant throughout, while there is a tendency for the corpuscle averages to decrease and those of whole blood to increase.

The average percentage of red blood cells dropped from 55 to 41.9 during the 12 day period.

To Dr. Bradford F. Dearing of the hospital staff I wish to express my sincere thanks for his interest and cooperation throughout this investigation.

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OBSERVATIONS ON BLOOD FAT IN DIABETES.

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(Received for publication, August 30, 1921.)

The former conception that diabetes is the result of a deranged carbohydrate metabolism is now known to be only a partial truth. It is today recognized that the metabolism of carbohydrate is faulty; also the utilization of fat and protein.

The metabolism of fat in this condition is particularly interesting and any study of the factors entering into the incomplete combustion of fats leading to ketonuria is desirable. As a result of the investigations of Bloor (1916), and of Joslin, Bloor, and Gray (1917), the extent of the increase of blood lipoids in diabetes has been demonstrated. Their data revealed that the greatest increase was in total fatty acids, secondly in cholesterol, and lastly in the lecithin fraction. This pioneer work with the satisfactory method of Bloor is of fundamental importance in arriving at a better understanding of the behavior of fats in diabetes.

Newburgh and Marsh (1920) have recently suggested a new method of treatment of diabetes, designed to overcome the undernutrition incident to the Allen and other similar diets. Accordingly, a low protein, low carbohydrate, high fat diet is advocated by these authors who reported seventy-three cases to have been successfully treated by this method. Their criteria of success were (1) a sugar-free urine; (2) no acidosis; (3) nitrogen balance maintained; and (4) capability of resuming the ordinary activities of life. Their diet is so constructed that it contains about 0.66 gm. of protein per kilo of body weight. This system although revolutionary in character, offered obvious desirable features if workable, and has been given a thorough trial in this Clinic. If the high fat diet does in fact allow the patient to be free from the acetone bodies, this should also be reflected in a constancy of values for blood fat.

Methods.

Total blood fat (fatty acids plus cholesterol) was determined by the method of Bloor (1917), the methods of Folin and Wu

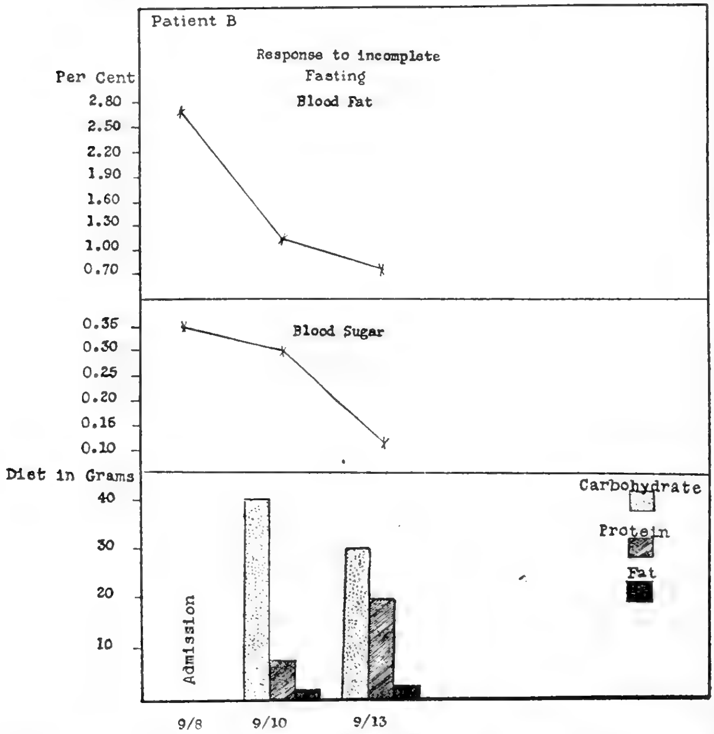

Chart 1. Male, 22 years of age. Sugar discovered in urine July, 1920. Best weight 145 pounds: now (Sept. 8, 1920) 79 pounds. Admitted on verge of coma. Sept. 8, 1920, he excreted 111 gm. of sugar. The urine of this date showed a + + + acetone and + diacetic reaction. The urine of Sept. 12 contained 18.6 gm. of sugar and was negative for acetone bodies. Lipemia was present in the first blood drawn.

(1920), and later of Shaffer and Hartmann (1920–21) were used for blood sugar determinations. All blood samples were taken in the postabsorptive condition before breakfast.

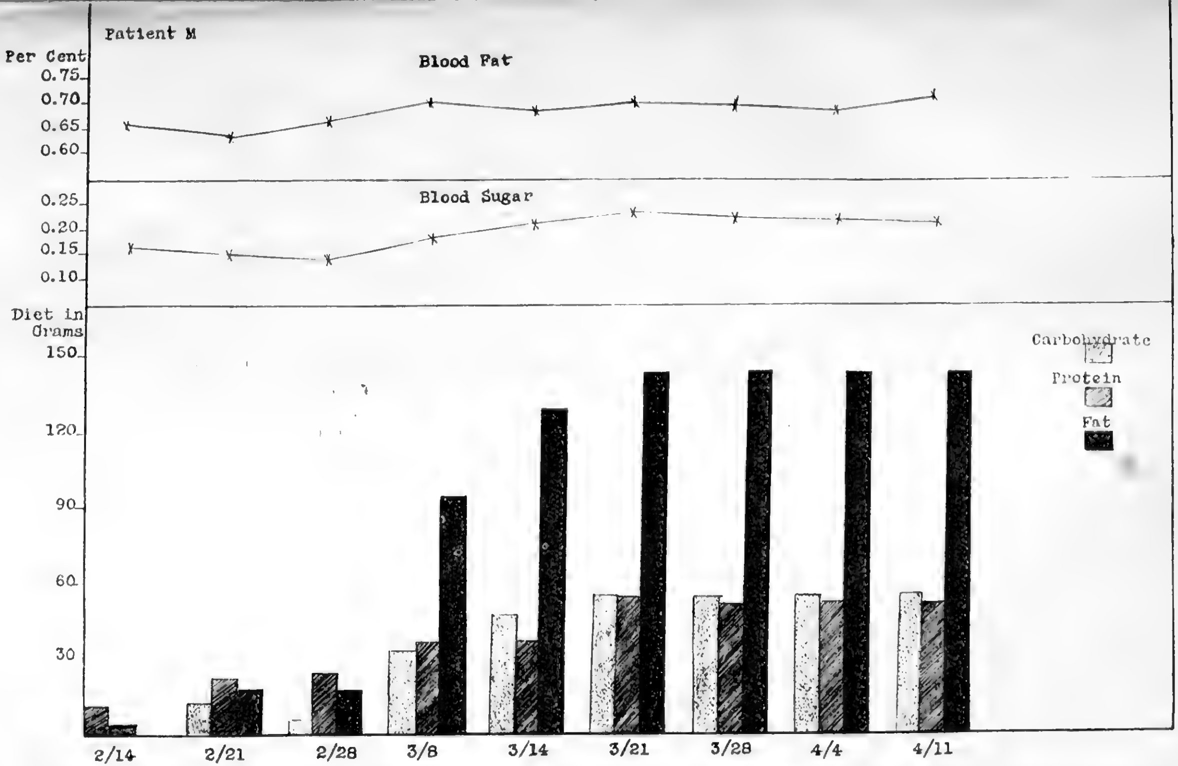

CHART 2. Male, 64 years old. Diabetes of 6 years duration. Loss in weight from 208 to 110 pounds. When admitted Feb. 10, a 12 hour specimen of urine contained 6.2 per cent glucose (44 gm.). This patient excreted sugar continuously, varying in amount from a trace on Feb. 28 to 14 gm. on Mar. 21. On Apr. 11, he excreted 6.2 gm. During most of the period traces of acetone were present. Blood fat was in no case greatly above normal. The patient was very hard to desugarize. He has been sugar-free since June 10, and is now (Aug. 15) eating 18 gm. of carbohydrate, 66 gm. of protein, and 157 gm. of fat daily. On this diet there is neither glycemia nor excretion of acetone bodies.

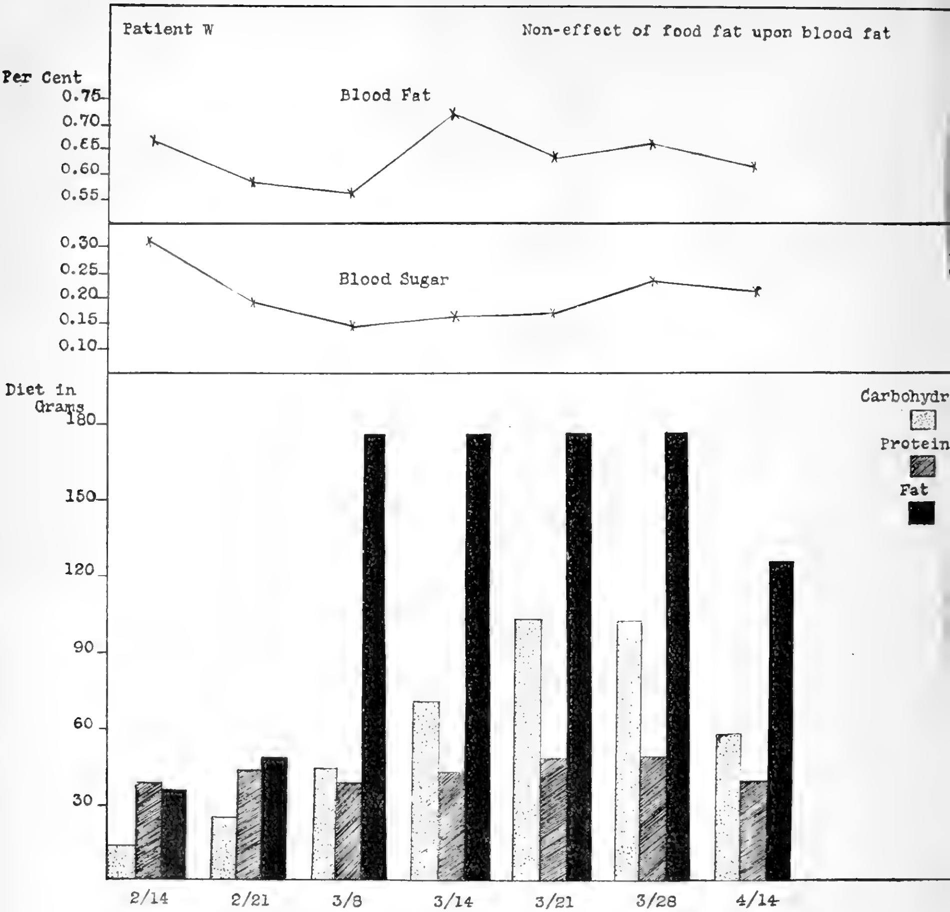

Chart 3. Male, age 49. Diabetes complicated with chronic pulmonary tuberculosis, chronic arthritis, and chronic nephritis. Diabetes of 4 years duration, with resultant loss in weight from 194 to 120 pounds. When admitted Feb. 10, a 12 hour specimen of urine contained 7.1 per cent glucose (119 gm.). The sugar excretion of this patient was 6.1 gm. on Feb. 14. He was sugar-free thereafter. A + + + acetone reaction was obtained on the above date and none or mere traces afterwards. Blood fat values in this case are also not much above normal.

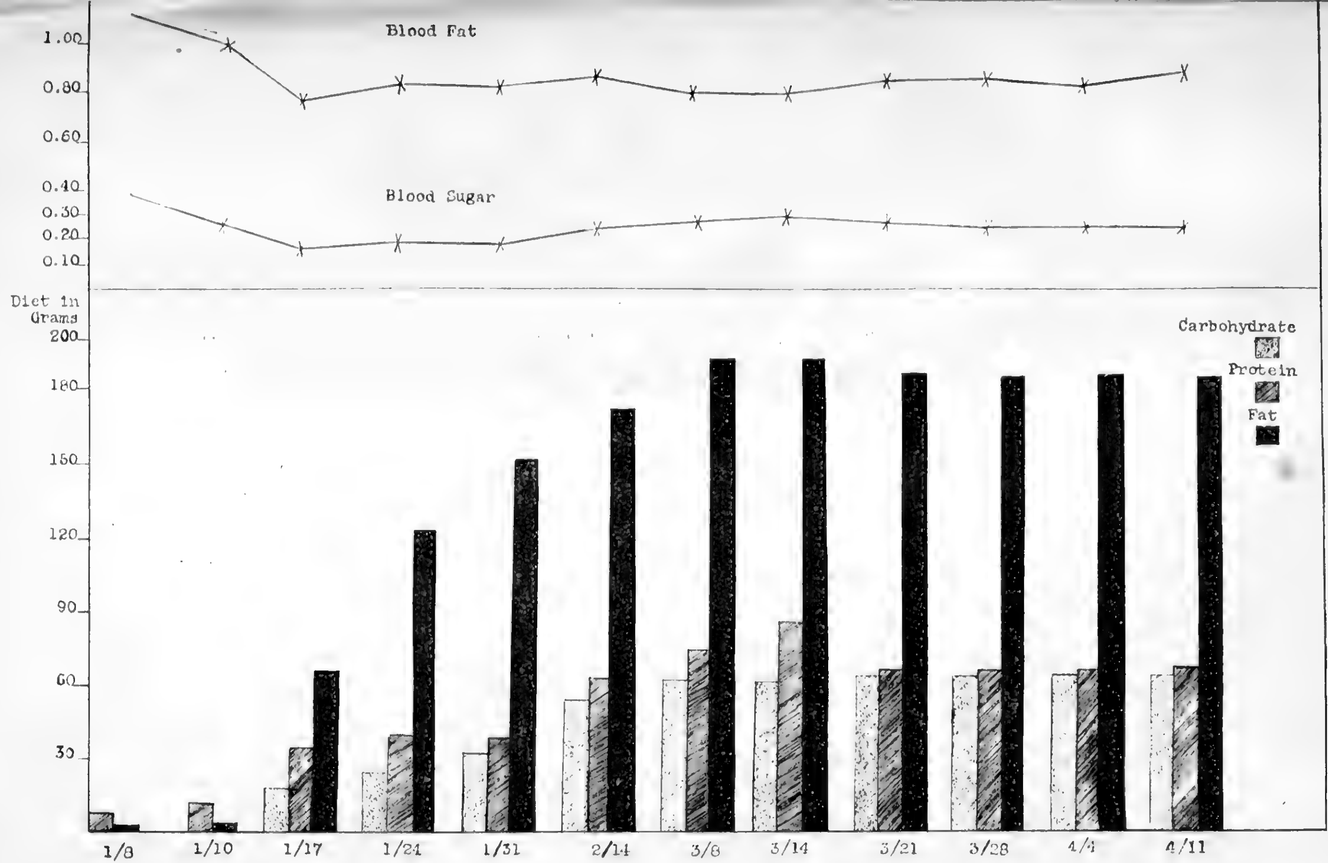

Chart 4. Female, nurse, age 41 years. Diabetes of 7 years duration. Loss in weight from 195 to 120 pounds during this period. Bilateral diabetic cataracts removed in two operations during interval covered by chart. Admitted Jan. 7. The urine of Jan. 8 contained 5.9 per cent sugar and a trace of acetone. After Jan. 10, no sugar was found and mere traces of acetone until Feb. 14. None was recorded during the remainder of the period.

Explanation of Charts.

In the charts are given percentage values for blood fat and blood sugar, and the diet in grams of carbohydrate, protein, and fat for the day preceding the blood sample. The diet was often constant over a long period, as will be seen by reference to the charts. Any supplementary data will be found in the legend of each chart.

RESULTS.

Chart 1 shows the response of a severe diabetic to partial fasting, such a restriction in diet as is generally prescribed when a patient exhibits marked ketonuria and lowered alkali reserve. In the course of 5 days, the values for blood fat and blood sugar are seen to have nearly reached the normal levels. The CO_2 capacity on admission was 26.5 cc., which became 74.8 cc. per 100 cc. of plasma on the last day charted. The drop in blood fat here found is not always to be expected. Bloor (1914) showed that the blood fat of fasting dogs may decrease or increase, depending upon the original nutritive condition of the animal. Probably the result here shown is the ordinary response of a hyperlipemic individual to such a restriction in diet.

Charts 2, 3, and 4 show the behavior of blood fat and blood sugar of patients living on diets similar to those recommended by Newburgh and Marsh. The outstanding feature is the constancy of values for blood fat with increasing amounts of fat in the diet. This indicates that these patients were utilizing the large amounts of fat in a satisfactory manner.

SUMMARY.

A study of blood fat in relation to the fat in the diet shows that cases of mild and moderate diabetes are apparently able to utilize satisfactorily large amounts of fat, as indicated by constancy of the blood fat level and by the absence of acetone bodies in the urine. It remains to be learned whether or not such high fat diets continued for a considerable period will prove to be an overstrain on the fat burning mechanism.

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LIPEMIA.

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The blood plasma in the postabsorptive period is normally clear although containing appreciable amounts of material of a fatty nature which is insoluble in water—cholesterol, cholesterol esters, lecithin, and probably also some fat. The introduction of more fat into the blood, whether by feeding or by mobilization from the fat stores, generally produces a milkiness normally lasting only a few hours, but which in certain conditions, mainly pathological, persists for considerably longer periods. The milkiness of the plasma is termed lipemia. In human beings the plasma contains normally 0.5 to 0.8 gm. of lipoid per 100 cc. and lipemia ordinarily appears when values above these figures, but generally below 1 gm. per 100 cc., are reached. In diabetics, values above 1 per cent with clear plasma are often found (1) and occasionally remarkably high values are reached, as in Case H in Table I, where a value of 4.35 gm. of total lipoid per 100 cc. with clear plasma was found. Allen (2) has recently reported a case of hepatic cirrhosis with "total fat" values of 3.63 per cent with clear plasma, and similar cases of "masked" lipemia have been frequently noted in the literature (3). The masking may be an unstable condition, since on standing for a time (24 to 48 hours) milkiness may develop in a plasma which was clear when drawn.[1] This latter phase of the subject, although interesting, has been very little investigated and will not be dealt with in the present discussion, which will be confined to lipemia as defined above—a more or less prolonged milkiness of the plasma.

Alimentary Lipemia.

During the absorption of fat from the intestine the fat of the blood of most animals increases and eventually a more or less

[1] Bloor (1), p. 429.

pronounced lipemia is produced, the extent of which depends on several factors, of which the most important are probably the rate of absorption from the intestines and the rate of disappearance from the blood. The rabbit is unique among the animals investigated in that lipemia apparently cannot be produced in it by fat feeding (4). There can be no question about the absorption of fat in these animals and the explanation of the non-appearance of lipemia must lie in the fact that the rate of disappearance from the blood is equal to or greater than the rate of absorption. In most animals if the increase of fat in the blood is considerable, it is followed by increases in the other blood lipoids. There is an increase of lecithin (5 to 8) and sometimes also of cholesterol (6), although the latter is not always found (7), which may be explained by the observation that the increase in cholesterol often does not appear till late in the period of absorption (9). There appears to be a definite sequence in the changes in the three lipoids in the blood during fat absorption—the presence of fat if sufficiently large in amount or sufficiently persistent causing an increase of lecithin, and this in turn is followed by an increase in cholesterol. However, as was pointed out by Bang (10), animals vary a great deal both in their reaction to ingested fat (production of lipemia) and in the effect which fat in the blood has on the other blood lipoids, and it is not always possible to demonstrate these changes. Hueck and Wacker (11) have found that prolonged feeding of cholesterol may also produce a lipemia (rabbits) with secondary increases of lecithin and fat.

In the study of the blood in alimentary lipemia, examination in most cases has been confined to the plasma but in the few instances where the distribution of the lipoids between plasma and corpuscles has been studied (8) increases of lecithin and fat have been found in the corpuscles as well, in fact the main increase of lecithin in the blood has been found there. This participation of the corpuscles in the utilization of fat appears to be only temporary since in the persistent lipemia noted below the corpuscles appear to have little or no part.

Persistent Lipemia.

The lipemia produced by a single fat feeding normally disappears within 24 hours and persistence after this time is prob-

ably to be regarded as pathological. (This statement cannot apply, of course, to instances where the ingestion of fat is more or less continuous—as in suckling animals or in animals subjected to forced feeding for fattening or other purposes, in both of which the lipemia may be continuous.) By far the greatest number of cases of persistent lipemia have been found in diabetes, although nephritis and chronic alcoholism furnish some examples. In this condition when examinations of lipoids other than fat have been made it was found that both lecithin and cholesterol were increased along with the fat—that there was a "lipoidemia" along with the lipemia (12). Since the discovery by Boggs and Morris (13) that a high grade lipemia can be produced in rabbits by bleeding, considerable study has been devoted to this type of lipemia and the data obtained are of especial value since the lipemia has been followed throughout its whole course (14, 15). In hemorrhagic lipemia also, all the blood lipoids are found to be increased.

The purpose of the present paper is to present additional data on the blood lipoids in persistent lipemia especially that of diabetes and of hemorrhage, and to discuss this and earlier data with the idea of reaching some conclusions as to the mechanism and probable cause of persistent lipemia, and its relation to normal fat metabolism.

The Lipemia of Diabetes.—In diabetes, under the older forms of treatment, persistent lipemia was a common phenomenon and it was early recognized that the milky appearance of the plasma was due to "fat." As to the nature of this fat very little was known until the beginning of the present century when Fischer (3) found that it contained abnormally large amounts of cholesterol, a result which Klemperer and Umber (16) confirmed, and noted in addition that the content of lecithin was also high. Investigators since that time have in general confirmed these findings although not always (17). Examination of the blood of diabetics without visible lipemia (blood plasma clear) has shown that all the blood lipoids are generally abnormally high especially in the severer cases (1), so that the diabetic may be said to be predisposed to that disturbance of fat metabolism of which the visible sign is lipemia. Up to the present time the data on blood lipoids in diabetes have been obtained by the

analysis of single samples and no attempt has been made to follow the course of the lipemia by examination of repeated samples taken during the course of the lipemia. It was felt that such a study would be of value not only from its relation to fat metabolism but also as a basis for the rational treatment of such abnormal conditions. Satisfactory material for examination is not readily obtained and the author is greatly indebted to Drs. E. P. Joslin and A. A. Hormor of Boston for supplying not only blood samples taken under necessarily exacting conditions but also for furnishing other data of interest. The methods used were the published ones for total fat, cholesterol, and lecithin, and need not be given here. The results of the analyses are given in Table I. For purposes of comparison the normal average values for blood lipoids in man are given at the top of the table also, and in the case of Subject Cl, the analysis of a blood sample taken some time previously when his blood plasma was clear.

Discussion of Table I.—The first case presented is the most interesting, both because the lipemia is the most pronounced and because it has been more extensively studied than the others. The patient was a severe diabetic, who had been under observation and careful dieting for some time. On June 10th he broke diet, eating among other things large amounts of milk and cream. On June 12th the first sample of blood was taken, the patient meantime having taken no food since the time of breaking diet. In this first sample is to be noted the very high values for all the lipoids, the total lipoid being approximately eight times his own normal value and fourteen times the average for normal men, while lecithin is approximately four and cholesterol eight times the values found in normal men. The greatest increase is thus in the fat, as has always been found to be the case in lipemia. Other notable points are the low corpuscle percentage (diabetic anemia) and the low values for the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$. The high urinary ammonia and the low carbon dioxide tension in the alveolar air are also significant. (The data in the last column were supplied by Dr. Joslin.) The next two samples taken June 14th and 15th show higher values for the lipoid constituents, the total lipoid value on the latter date reaching its maximum of

about 13 per cent. Whether these higher figures represent an actual increase in the fatty matter of the blood (which could come only from the fat stores, since no fat and very little food of any kind was taken), or whether it is due to a concentration of the plasma is open to question, but might well be the latter, since the blood corpuscle percentage in the last sample was 39, an increase of about 33 per cent over that of the first sample taken, (29 per cent). The lecithin in the last (third) sample is five times, cholesterol nine times, and total lipoid twenty times the values for normal men. In succeeding samples the total lipoid (plasma) diminishes rapidly, the lecithin less rapidly, and the cholesterol relatively slowly, showing that the fat is the first to decrease as it was first to increase, the lecithin next, and the cholesterol last. In the last sample examined, taken about a month after the beginning of the lipemia, the lecithin value was still about 30 per cent, the cholesterol about 60 per cent above, and the total fatty acid over 100 per cent above the normal values for this man. Although the plasma was now clear, calculated values for fat (fatty acid not combined as lecithin or in cholesterol esters according to the usual assumptions) in the plasma of the last sample gives a value of over 0.5 per cent which, since that amount of free fat would result in milkiness, indicates that the fat or fatty acid exists in the blood in some form of combination other than those ordinarily believed to be present. $\frac{\text{Lecithin}}{\text{Cholesterol}}$ in plasma is also much above the normal due to the slow rate of disappearance of the cholesterol. The addition of fat to the diet toward the end of the lipemia and increasing its caloric value did not cause any increase in the lipemia (with the possible exception of cholesterol) which continued to diminish about as before.

In the second person studied (Subject Sc) much the same phenomena are observable. The beginning high values for total fatty acids, lecithin, and cholesterol diminish slowly during the 3 weeks of observation; the values for the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ remain (except in Sample 566) much below normal, and the calculated value for fat in the clear plasma of the final sample (about 0.5 per cent) indicates as before the presence of unknown compounds

TABLE I.

Diabetic Lipemia.

No.	Date.	Corpuscles.	Total fatty acid per 100 cc.			Lecithin per 100 cc.			Cholesterol per 100 cc.			Total lipoid per 100 cc.	Lecithin/Cholesterol		Remarks.
			Whole.	Plasma.	Corpuscles calculated.	Whole.	Plasma.	Corpuscles calculated.	Whole.	Plasma.	Corpuscles calculated.	Plasma.	Whole.	Plasma.	
Normal average.															
		per cent	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
			360	380	360	300	220	400	210	220	190	670	1.44	0.96	Normal average for men.
Subject Cl.															
42	Jan. 4	42	440	460	410	440	380	530	370	480	220	1,100	1.18	0.80	Normal values for this man. Broke diet June 10.
124	June 12	29	6,100	7,000	4,000	920	840	1,300	1,260	1,600	420	8,850	0.73	0.53	Second day after food. Plasma creamy; diacetic, ++; NH_3, 3.9; blood sugar, 0.27; alveolar CO_2, 24 mm.
125	" 14	32	6,200	7,200	4,000	920	810	1,150	1,300	1,800	240	9,230	0.70	0.45	Plasma creamy; blood sugar, 0.3; NH_3, 3.1; alveolar CO_2, 35; diacetic, trace.
127	" 15	39	7,500	10,800	2,340	900	1,110	800	1,300	1,900	290	13,100	0.70	0.53	Plasma creamy; NH_3, 2.02; diacetic, slight.

128	June 20	35	3,500	4,700	1,270	640	620	660	1,050	1,460	300	6,360	0.61	0.43	Plasma thick, milky; diacetic, 0; alveolar CO_2, 31.
130	" 22	39	2,050	2,700	1,030	600	640	530	950	1,350	320	4,260	0.63	0.48	Plasma milky; diacetic, 0; alveolar CO_2, 32.
136	" 28	32	1,230	1,280	1,120	530	570	450	750	940	340	2,390	0.70	0.60	Plasma thin, milky; diacetic, 0; alveolar CO_2, 31; combined cholesterol, 56 per cent (normal).
138	July 3			1,300			590			760		2,230		0.77	Plasma faintly milky; diacetic, 0.
140	" 6	36	1,030	1,200	730	450	530	350	650	800	380	2,160	0.70	0.66	" opalescent; blood sugar, 0.13; combined cholesterol, 56 per cent.
141	" 10	33	870	1,160	310	460	510	260	570	760	210	2,020	0.80	0.67	Plasma clear; blood sugar, 0.18.

Subject Sc.

561	May 22	37	1,370	1,300	1,370	640	700	540	930	1,300	310	2,850	0.68	0.54	Plasma milky, translucent; blood sugar, 0.2.
563	" 25	42	970	1,580	850	520	690	400	900	1,520	480	3,220	0.58	0.45	Plasma milky; 2 days on fat-free diet; blood sugar, 0.13.
566	" 31	37	960	1,230	500	540	660	340	760	770	730	2,230	0.71	0.90	Plasma slightly milky, 30 gm. of fat for 2 days; blood sugar, 0.13.
568	June 4	41	1,000	1,430	400	550	550	550	640	870	325	2,490	0.86	0.63	Plasma slightly cloudy.
571	" 8	38	1,170	1,600	450	590	540	660	630	850	270	2,670	0.94	0.64	" " " blood sugar, 0.16.
574	" 12	45		1,080			550			820		2,090		0.67	Plasma clear.

TABLE I—*Concluded.*

No.	Date.	Corpuscles.	Total fatty acid per 100 cc.			Lecithin per 100 cc.			Cholesterol per 100 cc.			Total lipoid per 100 cc.	$\frac{\text{Lecithin}}{\text{Cholesterol}}$.		Remarks.
			Whole.	Plasma.	Corpuscles calculated.	Whole.	Plasma.	Corpuscles calculated.	Whole.	Plasma.	Corpuscles calculated.	Plasma.	Whole.	Plasma.	
Subject Ra.															
		per cent	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
562	May 23	36	730	1,030	250	430	440	420	470	530	360	1,680	0.90	0.83	Plasma milky.
564	" 27	43	460	530	260	370	380	350	420	550	250	1,220	0.90	0.66	" faintly milky; 3 days fat-free.
567	" 31	38	380	550	110	340	310	390	380	500	180	1,150	0.90	0.62	Plasma clear and bright.
Subject Co.															
123	June 10	43	1,300	2,100	235	300	320	280	500	700	230	2,910	0.60	0.46	Plasma milky.
Subject H.															
578	Oct. 20	32	2,750	3,500	1,100	700	640	816	1,330	1,710	850	5,500	0.53	0.37	Plasma milky.
579	" 25	34	2,470	2,960	1,950	848	1,120	400	1,330	1,540	510	4,870	0.64	0.72	" thin, milky.
580	" 29	34	2,410	2,830	1,940	760	1,060	190	1,290	1,500	530	4,720	0.59	0.70	" faintly opalescent.
583	Nov. 5	30	2,130	2,630	1,370	980	1,020	230	1,170	1,370	300	4,350	0.84	0.75	" clear.
584	" 16	31	2,820	3,550	1,200	824	920	590							" faintly cloudy, almost clear.

of fat or fatty acid. A similar parallelism between corpuscle percentage (concentration of plasma) and lipoid values as in Subject Cl may be noted here also—when the corpuscle percentage is low (dilute plasma) the lipoid values tend also to be low, and *vice versa.*

In the third person studied (Subject Ra) the total fatty acids and lecithin diminish regularly in the week of observation, the lecithin more slowly than the fatty acid, while the cholesterol remains practically unchanged, resulting in regularly diminishing values for the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$.

The fifth case is remarkable because of the very high lipoid values throughout. Even when the plasma is clear as in the last sample, the total lipoid content is over 4 per cent, an extraordinary value for clear plasma, which is, however, paralleled by the values for lecithin (1 per cent), cholesterol (1.4 per cent), and to a less extent by the calculated value for fat (1.5 per cent). In the period of observation, lasting about 2 weeks, the lecithin increased after the first sample and remained constant at the high value while the cholesterol, beginning high diminished slightly. This person differs from the others and especially from Subject Cl in that the lipemia was probably of long standing.

The values given in Table I for the corpuscles are calculated from values for whole blood and plasma, taking into account the corpuscle percentage. They are, therefore, probably less accurate than the directly determined values and great emphasis cannot be placed on them. Such as they are, however, they indicate that the corpuscles take some part or are to some extent affected by the changes in the lipoid values of the plasma. The changes in value are much less marked and lasting than those of the plasma, although too great to be accounted for by small amounts of plasma existing as impurity in the corpuscle mixture after separation. On the other hand, they do not indicate that the corpuscles take the active part in lipoid metabolism observed in alimentary lipemia.

The Lipemia Produced by Hemorrhage.—That lipemia may be produced by extensive bleeding was first demonstrated by Boggs and Morris in rabbits (13). The amount of bleeding necessary and the time required to produce the lipemia varied with dif-

ferent animals but the end-result was always a pronounced milkiness of the plasma with total lipoid values up to 4.5 per cent (about twenty times the normal value for rabbits). They found the lecithin values to be much increased, but not the cholesterol. The fatty acids contained in the lipoids were of high iodine value (I. N. = 105). Sakai (4) repeated and confirmed the findings of Boggs and Morris but found the cholesterol values also much increased. What is of further interest in connection with Sakai's work is that while, as reported in the literature, alimentary lipemia could not be produced in normal rabbits (a finding confirmed by many experiments in this laboratory), it could be demonstrated in certain of the rabbits from which 13 to 20 cc. of blood had been drawn for 5 successive days. Horiuchi (14) has recently contributed extensive and detailed data on this type of lipemia making use of methods requiring but small amounts of blood, so that the whole progress of the lipemia, at least as regards changes in the blood lipoids, was studied day by day from its rise to the return of the lipoid values to normal. Since his work furnishes the best picture of the condition at present available, it is worthy of more extended comment. As the result of his experiments Horiuchi concludes that (1) the lipemia is never a phenomenon of fat alone—there are always corresponding increases of cholesterol and lecithin, which, however, are never as great as those of the fat; (2) the actual lipemia, in the sense of milkiness of the plasma, is of relatively short duration, but high values for cholesterol and lecithin persist for a longer time. Thus in Rabbit A, plasma values for lecithin and cholesterol continued above normal for 6 days after the plasma was clear of visible fat. (3) Increases in lipoid values in the corpuscles are relatively small although they may persist for a long time. Thus in Rabbit A, high values for lecithin and cholesterol in the corpuscles persisted for 16 days after the plasma had become clear. (4) The lipemia occurs whether the animal is on a low or a high fat diet but is produced more readily and lasts longer on the high fat diet. Further observations that may be made on these experiments are that the general sequence of increases of the lipoids is first fat, then lecithin, and finally cholesterol, and in the decrease, the fat diminishes first, the lecithin next, while the high cholesterol values persist longest. In most

of his experiments the values for the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ decrease as the experiment proceeds, although in some experiments the early values may be above the beginning value, and in others the value for the ratio is higher throughout the experiment than at the beginning, which would indicate that there may be a limit to the possibility of increase of cholesterol. Feigl (15) began a study of the same condition using the same methods but his work was interrupted by the publication of Horiuchi's results. He contributes a study on three animals with results that confirm those of Horiuchi. In addition he presents data on blood lipoids in several cases of extensive hemorrhage in human beings in which he finds similar changes in the blood lipoids (see further reference below).

As part of a study of the effects of severe hemorrhage on rabbits made for another purpose (18) examination was made of the blood lipoids in the lipemia produced. A similar study was made on dogs under the same conditions but with the result that in three experiments with extensive bleeding it was found impossible either to produce lipemia or even to change appreciably the blood lipoid values. Results of four of the rabbit experiments and of the one dog experiment in which even slight changes in the blood lipoids could be produced are given in Table II. The methods used were for lecithin the same as in the previous work, for total fatty acids and cholesterol a new method, shortly to be published, by which separate and direct determinations of these two substances are made. The values for cholesterol by the new method are practically the same as by the older saponification methods, those for total fatty acids are considerably lower than by the old indirect method. The values for corpuscles are by direct determination instead of by calculation as in Table I.

Discussion of Table II.—The results of the rabbit experiments confirm the conclusions of Horiuchi in most points. In the plasma there is always a lipoidemia accompanying the lipemia and it persists for some time after the visible lipemia (milkiness of plasma) has disappeared. In all the experiments the plasma lecithin increases before the cholesterol. The values for the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ are less at the height of the lipemia than at the

TABLE II.

Blood Lipoids in Hemorrhagic Lipemia.

Sample No.	Date.	Corpuscles.	Bleeding.	Total fatty acid per 100 cc. Plasma.	Total fatty acid per 100 cc. Corpuscles.	Lecithin per 100 cc. Plasma.	Lecithin per 100 cc. Corpuscles.	Cholesterol per 100 cc. Plasma.	Cholesterol per 100 cc. Corpuscles.	Total lipoid per 100 cc. Plasma.	$\frac{\text{Lecithin}}{\text{Cholesterol}}$. Plasma.	$\frac{\text{Lecithin}}{\text{Cholesterol}}$. Corpuscles.	Remarks.
Rabbit A. Weight 3.75 kilos.													
		per cent	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
1	Oct. 1	36.0	30	220		75		13		258	5.8		Plasma clear.
2	" 2	28.1	30	220	450	80	640	13	140	261	6.1	4.6	" "
3	" 3	21.5	30	300		90		23		353	4.0		" "
4	" 4	19.3	30	340		190		70?					" somewhat milky.
5	" 5	18.0	30	700		210		45		815	4.7		" " "
6	" 6	16.9	30	830	600	210	1,120	50	170	940	4.2	6.6	" milky.
7	" 7	16.5	30	670	600	240	1,250	80	170	830	3.0	7.3	" very milky.
8	" 8	16.1	15	860	630	240	1,200	110	170	990	2.2	7.0	" " "
9	" 9	19.5	15	640	800	310	1,280	100	200	844	3.1	6.4	" " "
10	" 10	20.0	15	350	740	300	1,280	83	130	533	3.6	9.8	" milky.
11	" 11	21.0	15	140	640	260	930	75		360	3.4		" clear.
15	" 16	26.5	15	160	670	80	960	25	200	215	3.2	4.8	" " 15 cc. samples blood were taken Oct. 12, 13, and 14.
17	" 17	30.1	15	190	640	112	960	22	160	244	5.0	6.0	Plasma clear.
27	" 27	34.4	15	80	650	64	760	10	170	110	4.5	6.4	" "

Rabbit B. Weight 3.9 kilos.

1	Oct. 21	34.4	30	170	600		670	56	130			5.1	Plasma clear.
2	" 22	23.3	30	100	430	64	640	34	200	156	1.9	3.2	" " $\frac{L}{C}$ low to start with.*
3	" 23	20.7	30	120	220	64	640	42	200	186	1.5	3.2	" "
4	" 24	18.4	30	230	350	88	870	42	80	302	2.1		" faintly milky.
5	" 25	17.8	30	480	500	160	720	44	140	570	3.6	5.1	" moderately milky.
6	" 26	19.6	30	590	650	190	1,080	45	140	699	4.2	7.6	" " "
7	" 27	17.3	30	950	660	240	970	60	145	1,090	4.0	6.7	" milky.
8	" 28	17.5	30	1,000	660	300	1,160	100	140	1,210	3.0	8.0	" "
9	" 29	18.0	30	950	800	260	1,300	90	190	1,128	2.9	6.9	" "
10	" 30	16.1	15	950	670	340	1,200	90	190	1,155	3.8	6.3	" "
11	" 31	17.7	15	670	640	180	1,120	56?	160			7.0	" "
12	Nov. 1	20.2	15	950	800	260	1,200	85	140	1,130	3.0	8.0	" "
15	" 6	25.7	15	440	600	150	1,300	50	140	540	3.0	9.0	" faintly milky
19	" 11	25.8	15	130	500	64	1,360	17	140	167	3.7	9.7	" clear.
37	" 29	36.5	15	60	240	56	600	16	90	94	3.5	6.6	" "

Rabbit C. Weight 3.4 kilos.

1	Mar. 16	35.5	20	106	235	60	504	23	110	149	2.6	4.6	Plasma muddy.
2	" 17	30.3	33	100	230	58	480	23	125	142	2.5	3.8	" "
4	" 19	23.0	25	125		80		24		174	3.3		" cloudy, lipemia? 25 cc. blood taken Mar. 18.
5	" 20	18.4	25	104	320	82	510	27	150	159	3.0	3.4	Plasma slightly muddy.
6	" 21	18.1	27	255		89		44		328	2.0		" faintly milky.
7	" 22	18.1	30	230	320	112	600	44	145	312	2.5	4.1	" " "
8	" 23	16.5	45	530		136		100		676	1.36		" somewhat milky.
9	" 24	19.6	30	530	340	160	800	88	155	642	1.8	5.1	" milky.
10	" 27	28.7	20	123	270	80	1,000	50	160	198	1.6	6.2	" faintly milky.
11	Apr. 3	34.5	18	66	280	56	800	18	145	100	3.1	5.5	" clear.

* $\frac{L}{C}$ indicates $\frac{\text{lecithin}}{\text{cholesterol}}$.

TABLE II—*Concluded.*

Sample No.	Date	Corpuscles.	Bleeding.	Total fatty acid per 100 cc. Plasma.	Total fatty acid per 100 cc. Corpuscles.	Lecithin per 100 cc. Plasma.	Lecithin per 100 cc. Corpuscles.	Cholesterol per 100 cc. Plasma.	Cholesterol per 100 cc. Corpuscles.	Total lipoid per 100 cc. Plasma.	Lecithin/Cholesterol Plasma.	Lecithin/Cholesterol Corpuscles.	Remarks.
Rabbit F. Weight 2.3 kilos.													
		per cent	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
1	Apr. 13	36.0	18		210	114	500		150			3.3	Plasma muddy with coarse suspension.
2	" 14	29.2	20	111		128		36		191	3.6		" " " " "
3	" 15	24.6	18	185	300	124	608	89?	145	316	1.4	4.2	" " " " "
4	" 16	22.5	20	300		160		70		424	2.3		" thin, milky.
5	" 17	21.3	20	256	320	172	832	85	150	400	2.0	5.5	" milky.
6	" 18	23.1	15	167		133		95		306	1.4		" thin, milky.
7	" 19	20.3	20	250	300	152	960	165?	170	467	0.9	5.6	" faintly milky.
8	" 27	30.8	14	140	340	89	800	40	175	209	2.2	4.6	" muddy.
Dog. Weight 17 kilos.													
1	Sept. 23	45.3	500	385	850	172	360	76	154	519	2.2	2.3	Plasma clear.
2	" 24	31.1	20	450	760	192	416	66	147	580	2.9	2.8	" "
3	" 25	30.3	320	455	780	200	456	66	130	589	3.0	3.5	" "
4	" 27	22.9	325	385	820	200	440	66	125	519	3.0	3.5	" "
5	" 28	18.2	300	384	654	224	432	70	147	530	3.2	2.9	" "
6	" 29	15.8	320	300	700	200	496	80	150	448	2.5	3.3	" "
7	Oct. 1	13.1	25	390	920	248	440	92	200	566	2.6	2.2	" very faintly milky.
8	" 4	12.9	25	330	1,000	216	528	92	220	494	2.3	2.4	" clear.
9	" 8	16.0	25	500	995	296	600	104	220	704	2.8	2.7	" very faintly milky.
10	" 13	20.4	30	500	900	288	600	104	200	700	2.7	3.0	" faintly milky.

beginning in three out of four experiments. In Rabbit B the values at the height of the lipemia are much higher than at the beginning, but in this case the values for the ratio were very low (for rabbits) at the beginning. In general much more marked increases in the lipoid values of the corpuscles are to be noted in these experiments than in those of Horiuchi. These are most marked in the lecithin, the cholesterol often showing relatively little change and the total fatty acid still less.

In the dog, even with a reduction of red blood corpuscles from 45 to 13 per cent of the blood, no definite lipemia could be produced and the only marked change in the blood lipoids to be noted was in the lecithin which was considerably increased in both plasma and corpuscles. Values for cholesterol and total fatty acids remained quite constant. It is significant that the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ is consistently above normal throughout the bleeding in the dog, while in the animals in which lipemia is produced by bleeding it is almost always below normal which relates the low values of the ratio to the lipemia and not to the bleeding. Another feature of importance is that lecithin is more markedly increased than fat or cholesterol which emphasizes a point observed in earlier (unpublished) work, that slight or transient increases of fat may bring about an increase of lecithin without any change in cholesterol.

In order to show more plainly the features characteristic of lipemia and to compare lipemia of different origin, data from this paper and from various other sources are collected together into Table III. Values for plasma alone are given since differences from the normal are most plainly seen there and the values chosen for comparison are those taken at the height of the lipemia. The value for the constituent in the normal condition, or in case this has not been determined, the normal value for the species, is taken as 1 and the values found are reported as multiples or fractions of 1.

Discussion of Table III.—A study of the data presented in Table III brings out the following regarding lipemia. (*a*) In all the types of lipemia there is always an increase of lecithin and cholesterol as well as fat. (*b*) At the height of the lipemia the greatest increases are to be noted in the fat, the next in the

TABLE III.

Relative Values of Lipoids at Height of Lipemia (Normal = 1).

Type of lipemia, case, and author.		Fat.	Lecithin.	Cholesterol.	$\frac{\text{Lecithin}}{\text{Cholesterol}}$.	Remarks.
			Human. Diabetic.			
Case Cl.	(Bloor)	26.0	5.0	8.6	0.5	
" Schn.	(")	4.4	2.1	6.9	0.46	
" Ra.	(")	2.9	2.0	4.4	0.88	
" Co.	(")	5.8	1.5	6.2	0.48	
" H.	(")	9.5	2.9	7.9	0.38	
			Human.			
Operation.	(Feigl)	11.0	2.0	2.2	0.9	$\frac{L}{C}$ at beginning of lipemia 1.3 falls continuously to 0.8.*
"	(")	18.0	5.6	5.7	0.93	$\frac{L}{C}$ at beginning 1.15 falls to 0.75.
"	(")	5.5	1.4	1.4	1.06	$\frac{L}{C}$ falls to 0.65.
Ulcers.	(Feigl)	10.5	2.3	2.3	1.04	
"	(")	5.5	1.8	1.9	1.0	
"	(")	5.0	1.45	1.45	1.04	
Menstrual.	(Feigl)	5.5	3.0	2.4	1.04	$\frac{L}{C}$ at beginning of lipemia 1.3, at end 0.84.
"	(")	11.3	3.8	2.9	1.14	$\frac{L}{C}$ falls to normal.
"	(")	7.4	3.3	3.0	1.1	$\frac{L}{C}$ rises to 1.14.

Tuberculosis. (Feigl)	5.3	2.3	1.9	1.3	$\frac{L}{C}$ at first 1.45.
" (")	1.5	2.0	2.0	1.04	
" (")	2.4	1.4	1.4	1.04	$\frac{L}{C}$ rises to 1.25 later (1 week).
" (")	2.8	1.1	1.1	1.04	$\frac{L}{C}$ falls to 0.85.
" (")	5.6	1.9	1.4	1.5	$\frac{L}{C}$ falls to 0.87 in 5 days.
		Rabbit.			
Experiment IA. (Horiuchi)	8.4	Fat diet. 3.6	4.0	0.91	$\frac{L}{C}$ remains below the beginning value throughout.
" IB. (")	15.5	Fat-free diet. 7.3	3.4	2.2	$\frac{L}{C}$ in 2 days drops to 0.7.
" IIA. (")	12.5	Fat diet. 4.6	6.5	0.82	
" IIB. (")	4.3	Fat-free diet. 2.6	2.3	1.09	$\frac{L}{C}$ falls to 0.9 at end.
" IIIA. (")	20.0	Fat diet. 7.0		1.08	
" $IIIA_2$. (")	26.0	Fat diet. 6.2	8.3	0.75	
" IIIB. (")	8.4	Fat-free diet. 2.5	2.0	1.26	$\frac{L}{C}$ normal next day.

* $\frac{L}{C}$ indicates $\frac{\text{lecithin}}{\text{cholesterol}}$.

TABLE III—*Concluded.*

Type of lipemia, case, and author.	Fat.	Lecithin.	Cholesterol.	$\frac{\text{Lecithin}}{\text{Cholesterol}}$.	Remarks.
Experiment 1 (Feigl)	10 0	4.5	6.0	0.69	$\frac{L}{C}$ returns to normal and remains.
" 2 (")	15.0	4.0	4.0	1.38	$\frac{L}{C}$ decreases to 0.9 in the next 2 days but increases again.
Rabbit A. (Bloor)	3.8	2.8	3.8	0.7	$\frac{L}{C}$ continues to become less until recovery.
" B. (")	6.0	4.7	1.8	1.6	$\frac{L}{C}$ in this animal very low to start with.
" C. (")	5.0	2.3	4.2	0.5	
" F. (")	2.7	1.4	1.9	0.6	
Guinea pig.					
Guinea pig. (Feigl)	10.0	4.0	7.5	0.5	$\frac{L}{C}$ remains low throughout.
Dog.					
Dog S4. (Bloor)	1.3	1.8	1.35	1.2	$\frac{L}{C}$ in corpuscles at this time is 2.3 times beginning value.

cholesterol, and the least in the lecithin. As a result of the greater increase of cholesterol the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ is generally below the normal value. In most of those cases where the ratio is above the normal value it sinks to normal or below as the lipemia continues. The outstanding characteristics of persistent lipemia of whatever origin are then: increases of lecithin and cholesterol along with the fat; and a greater increase of the cholesterol above that of lecithin resulting in a $\frac{\text{lecithin}}{\text{cholesterol}}$ ratio below the normal.

The study of lipemia from the time it begins until it subsides (Tables I and II) brings out in addition the fact that after fat, lecithin is the first lipoid to increase in most cases, and cholesterol the last; which makes it possible to correlate persistent or chronic lipemia with the temporary lipemia produced in normal animals by feeding fat, since in this type of lipemia the sequence of changes in the blood lipoids is also fat, lecithin, cholesterol. However, as pointed out above, there are many exceptions; for example, increases of cholesterol in alimentary lipemia have never been demonstrated in this laboratory, possibly because the experiments have been too short.

The Origin of the Fat in Persistent Lipemia.—Of the three possible sources, fat of the food, fat stored in the depots, and fat synthesized from other constituents of the food, the last may be eliminated from the discussion since we are entirely ignorant as to its amount or the conditions of its formation. With regard to the other two, both may be shown to be sources of the fat of lipemia and the importance of each varies with circumstances. In the examples of diabetic lipemia given in Table I, of the first and most striking it was known that the subject had consumed large amounts of fat just before the appearance of the lipemia and frequent previous examinations of his blood made it probable that there was no lipemia up to that time. Allen (2) has furnished evidence to show that in dogs made experimentally diabetic persistent lipemia may be produced by overfeeding with fat. The possibility that the fat in diabetic lipemia may also come from the fat stores cannot be denied but in view of the fact that severe diabetics are thin and have little

stored fat such an origin does not seem important. The older argument (12, 16) that the fat in diabetic lipemia must originate by cellular destruction, since the blood contains not only fat but lecithin and cholesterol, falls before the later observations that these lipoids increase with the fat in alimentary lipemia. The origin of the fat in diabetic lipemia appears to be mainly the fat of the food.

With regard to hemorrhagic lipemia, Horiuchi found that it may be produced on a diet practically free from fat and, therefore, the increased fat originated mainly in the fat depots, in which he is borne out by the observations of Boggs and Morris (13) that rabbits so treated become emaciated, and of Sakai (4) that in addition there was a marked fatty liver (which, however, is produced whenever there is excess fat in the blood from any source). The extra fat in the blood in hemorrhagic lipemia may thus originate mainly if not entirely in the fat stores. However, the work of both Horiuchi and Sakai make it plain that food fat when present may be equally effective in producing the lipemia.[2] The facts available thus point to the conclusion, which perhaps might have been expected, that persistent lipemia may be produced by fat from either the fat depots or the food.

The Origin of the Lecithin.—Because of its close chemical relationship to fat and the fact that it appears in the blood in alimentary lipemia there is little doubt that it is synthesized from fat and phosphoric acid.

The Source of Cholesterol.—No stores of cholesterol are known in the organism which would account for the increase in the blood in lipemia. Both cholesterol and lecithin are found in food fats but ordinarily in traces only, so that it seems likely that the cholesterol is also synthesized.

The Cause of the Lipemia.—An accumulation of fat in the blood could be brought about in only one way, an inflow greater than the outflow; and it must be decided in each case whether the

[2] One endogenous source of the extra fat in hemorrhagic lipemia which might be emphasized in this connection is the marrow of the long bones which in youth is largely a blood-forming organ (red bone marrow) but which in the adult consists mainly of fat. With the stimulus of the severe hemorrhage new blood-forming tissue would be formed, pushing the fatty tissue out into the circulation.

inflow is abnormally great or the outflow abnormally small. Sakai, who has discussed the lipemia produced by hemorrhage in rabbits, assumed that in these cases it is the outflow which is less than normal and produces evidence to explain why the outflow is diminished. The method which he uses to prove his point—change in surface tension as measured by the stalagmometric method in a solution of tributyrin and blood serum—is open to criticism when used for the purpose on the following points:

1. The presence in normal blood of amounts of lipase capable of splitting more than traces of fat in the period of time that fat remains in the blood in alimentary lipemia (8 to 14 hours) has never been satisfactorily demonstrated (19).

2. Tributyrin is so much more easily hydrolyzed than ordinary food fat that its use as a measure of lipase activity is open to question.

3. The assumption of a splitting as shown by changes in surface tension, although possibly correct, cannot safely be accepted without further evidence of hydrolysis—increase of free fatty acids, which Sakai was unable to obtain. Furthermore, as pointed out by Sakai himself, his most marked changes were obtained before any lipemia appeared, while when the lipemia was at its height, values obtained were in many cases but little below normal. There was no constant relation between the degree of lipemia and the drop number.

But quite aside from the acceptability of his evidence the question may be raised whether his assumption of a diminished outflow in hemorrhagic lipemia is correct. Turning to Horiuchi's tables, which give the best picture of the lipemia, in Table I B on the 9th day of the bleeding the total fatty acids in the plasma (which may be taken as the measure of the lipemia) jumps from 0.32 to 3.07, remains high for 3 days and then falls on the next day to 0.51, the hemoglobin and, therefore, the red blood corpuscle percentage remaining practically constant. In Tables II B and III B the same sudden rise and fall, but much less striking may be noted. In Table IV after a single large bleeding the plasma fatty acid rises from 0.25 to 0.66, is further increased by a second large bleeding to 1.24, and still further by a feeding of fat (sunflower seeds) to 2.15, but *the day following* it has fallen

to a third of this value (0.69) with a change in hemoglobin of only two points. In Rabbit A in Table II above the same sudden falling off, 640 to 350 to 140 mg. on successive days with corpuscle percentage of 19.5 to 20 to 21 may be noted. In the case of the results from the other rabbits of this table data on this interesting point are not available. The examples chosen are purposely those in which the rabbits were on a fat-free diet so that the clearness of the results is not interfered with by the presence of food fat, but the same rapid falling off, although not so marked, may be noted in his fat-fed rabbits where the data are sufficiently full. For example in his Table III A on the 4 successive days from the height of the lipemia the figures are 5.19 to 3.05 to 1.30 to 0.73. In the second part of this table the fall is from 5.35 to 2.25 in 3 days and from 2.25 to 0.38 in the next 3 days. The rapid fall in the blood lipoids does not bear out the assumption that the ability to remove fat from the blood is appreciably diminished in these animals and therefore renders gratuitous the explanation that the blood lipase is diminished by the bleeding. The fact pointed out by Sakai that an alimentary lipemia may be produced in rabbits which have been bled and not in normal rabbits, and which is supported by the data in Horiuchi's Table IV, may be explained by the probability that the fat from the food added to the large amount already in the blood exceeds the quantity which even a normal animal may dispose of. The rabbit normally is not a fat-eating animal and its ability to handle fat, being therefore probably small, may be relatively easily overstepped. In this connection the data on the dog are especially useful. The dog can normally eat and quickly dispose of large amounts of fat and it is therefore significant that it is not possible to produce an unmistakable lipemia in him even by a hemorrhage by which the corpuscles are reduced from 45 to 13 per cent as in Table II. Of further significance is the fact that in another of the dogs in which it was impossible to produce lipemia by bleeding, when the corpuscle percentage was at its lowest (reduced from 32 to 13 per cent), a feeding of 50 gm. of fat produced a normal reaction, plasma milky at 6 hours after feeding and clear the next day.[3]

[3] Unpublished experiment.

It appears incorrect, therefore, to assume that the lipemia produced by hemorrhage in rabbits is caused by a decreased outflow of fat from the blood since fat disappears from the blood of these animals with considerable rapidity and probably at not far from the normal rate. The lipemia may be equally well explained as due to the sudden discharge into the blood of quantities of fat larger than the normal mechanism can dispose of at once. Not knowing the normal rate of removal of fat from the blood it is, of course, impossible to exclude the possibility that there may be some slowing of outflow due to inanition of the tissues, etc., but in the lack of further data it is unnecessary to assume that the lipemia of hemorrhage in rabbits is due to anything more than larger influx of fat than the organism can take care of at once. As to the cause of the sudden mobilization nothing definite can be said. There is undoubtedly more or less cellular inanition which might cause a movement of stored food material, and the displacement of fat in the long bones by blood-forming tissue is an interesting possibility.

Turning now to the lipemia of diabetes, in the first example given in Table I the high beginning values (which were known to be due to a single intake of foot fat) persist unchanged for 3 days (the apparent *increase* in value being probably due mainly to concentration of the plasma), in 5 days more are only reduced to about half the highest value and milkiness of the plasma persists for 15 or 16 days longer. In the second example the high values and the milkiness persist for about 17 days and in the last example for about 14 days. Other examples of the slow disappearance of the lipemia in human diabetes are to be found in the literature (20). Allen (21) has shown that in suitably prepared diabetic dogs it is easy to produce a persistent lipemia while in the normal animal it is difficult or impossible. That lipemia is common in diabetes has been known for over a century (3) and it is the only disease in which it is at all frequent. Taking all the evidence together the assumption seems justified that in the diabetic the ability to remove fat from the blood is below normal and that herein is to be found the explanation of the lipemia of diabetes.

The commonly accepted theory of the cause of diabetes is the lack of a hormone normally found in the internal secretion of the

pancreas, and of which the function is the removal of excess sugar from the blood and its utilization or storing in the tissues. When this substance is lacking the excess sugar is lost in the urine. An extension of this theory to postulate the presence in the internal secretion of another hormone of which the function is to aid in the removal of fat from the blood has already been made (21) and has the following in its favor. First, the observation that persistent lipemia has always been commonest among diabetics; second, that lipemia may be readily produced in dogs made diabetic by removal of a suitable proportion of the pancreas, while its production in normal dogs is practically impossible; and third, that the blood lipoids in diabetics are almost always above normal, which may be regarded as evidence of diminished power to remove them. On the other hand, the deficiency of the hormone is probably never as great as that of the corresponding carbohydrate hormone since severe diabetics whose tolerance for carbohydrate is very low, do not ordinarily become lipemic, even with relatively large amounts of fat in their diet; in fact, they must depend largely on fat for their sustenance. Also, if it be taken for granted, as seems reasonable, that the appearance of persistent lipemia is equivalent physiologically to the appearance of sugar in the urine, the lack of the fat hormone in suitable amounts is relatively infrequent.

The factor of overwork must probably also be taken into consideration in examining into the cause of diabetic lipemia and possibly of other forms of lipemia. It is a biologic rule that a mechanism when worked within or up to the limit of its powers tends to become stronger, but when worked beyond its powers tends to fail. This principle is recognized in the treatment of diabetes by limiting the carbohydrate intake to an amount within the tolerance of the individual, in the expectation that by so doing the tolerance, or the amount which the individual may consume without waste, may be increased. It is well known that if this amount be exceeded the tolerance of the individual is likely to be lowered. There is very little evidence available to show that such is the case with regard to fat, but it seems probable. The most marked example of diabetic lipemia presented in this paper was an individual who both before and after the lipemia was able to deal with considerable amounts

of fat in his diet without lipemia, but when a large amount was taken, the mechanism for utilizing fat to all appearances broke down and did not recover for a considerable time.

It appears then that two types of lipemia are represented in the data given in this paper. One in which the ability to remove fat from the blood is little if any affected and in which the cause of the lipemia is to be sought rather in a flooding of the blood with amounts of fat greater than the normal mechanism can take care of at once. In this group are included the hemorrhagic lipemia of rabbits and probably also that of human beings, although enough data are not available to determine definitely about the latter. It is characterized by a rapid recovery as soon as the cause (the abnormal inflow of fat) is removed. In the other type, represented by the lipemia of diabetes, the cause of the lipemia is to be sought primarily in a diminished power to remove fat from the blood, referable to an inadequate supply of the pancreatic hormone, and secondarily to a temporary disablement of the fat burning mechanism due to overwork, which is recovered from rather slowly.

The behavior of lecithin and cholesterol in lipemia calls for some comment. It is probable that they have to do with the metabolism of the fat, since whenever the fat of the blood is increased they are also increased, and conversely, when the fat of the blood is low they are also low. In alimentary lipemia the changes follow the changes in the fat within a few hours. In persistent lipemia, although the increase of lecithin and cholesterol follows rather closely (within 2 days) the increase of fat, the values remain high after the fat has decreased to nearly normal and fall to near the normal value only after several days. Because of its close chemical relationship to the fats the change in lecithin calls for little comment, but why cholesterol, whose relationship to fat metabolism is rather remote (formation of esters with the fatty acids), should increase more or less parallel to the fat and lecithin is not readily apparent. Lecithin and cholesterol are antagonistic in several important reactions in the living body. Their opposite effect on tumor growth and possibly also on bodily growth and development in mice has been pointed out by Robertson and Burnett (22) and their antagonism in the matter of the stability of the red blood cells toward hemolysis seems well

established. It is possible that the increase of cholesterol in lipemia, which is in most cases not only equal to but generally greater than that of lecithin (the ratio of $\frac{\text{lecithin}}{\text{cholesterol}}$ is generally below normal), is to be considered a protective mechanism to counteract the effects of the increased lecithin. The writer is, however, of the opinion that this is only one and probably a less important function of cholesterol, and that others, particularly in relation to fat metabolism, will become apparent when more is known of the behavior of this mysterious substance in the organism. The slower rate of removal of lecithin and cholesterol as compared with fats points to a different mechanism for the removal of the two groups of lipoids.

SUMMARY.

The characteristics of persistent lipemia as shown by the evidence so far available are as follows:

1. In lipemia of whatever origin all three blood lipoids (fat, lecithin, and cholesterol) are increased, fat generally showing the greatest ultimate increase, and cholesterol next.

2. There is perceptible, in most cases, a sequence in the appearance and disappearance of the three lipoids, fat being the first to increase, lecithin next, and cholesterol last, while during the clearing up of the lipemia the fat diminishes first and the cholesterol last. High values for lecithin and cholesterol often persist for some time after the fat has reached approximately normal values.

3. In most instances the values for the ratio $\frac{\text{lecithin}}{\text{cholesterol}}$ are markedly below normal, due to the greater increase of cholesterol over lecithin.

4. The fat which produces the lipemia may be of endogenous or exogenous origin or both, but the phenomena of the lipemia are the same in either case.

5. The cause of the lipemia being regarded as a disturbance of the balance between inflow and outflow of fat in the blood, the immediate causative factor in the hemorrhagic lipemia is probably an abnormally large inflow of fat while in diabetic lipemia it is an abnormally slow outflow.

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STUDIES OF LIVER FUNCTION.

BENZOATE ADMINISTRATION AND HIPPURIC ACID SYNTHESIS.

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Much work has been done in an effort to understand the complex function of the liver and considerable information of value has been obtained. It is fair to say, however, that the estimation of functional capacity of the liver cannot as yet be made with any convincing accuracy. We have reason to believe that the liver has a very large factor of safety—that it can tolerate an extensive injury and yet carry on its essential body functions. Any satisfactory functional test must include, therefore, some factor of strain or load which can measure the upper limits as well as the lower limits of liver function. It is certainly possible that some of the many functions of the liver may be seriously impaired while others, perhaps of more fundamental significance, are not seriously disturbed. This may apply to acute as well as chronic disease conditions.

Probably the liver function test which is of most value in physiological experiments is given by the use of phenoltetrachlorphthalein as described by Whipple, Peightal, and Clark (1). This drug is given intravenously and eliminated promptly in the bile. It can readily be recovered from the feces and accurately estimated. This functional test shows a great impairment after extensive liver injury by chloroform or phosphorus, but a serious objection is at once obvious if any abnormalities of bile secretion are present. With complete biliary obstruction obviously the test is of little value and with chronic inflammation of the bile passages, we find a marked lowering in output of the phenoltetrachlorphthalein—for example in long standing biliary fistulas. For clinical use, therefore, this test has very serious

limitations. We cannot rely in clinical diagnosis on any liver test which postulates a normal bile flow in normal bile passages.

The ideal liver functional test would consist in the introduction intravenously of some non-toxic substance which would test by a synthetic demand the functional reserve of the liver (normal or abnormal). The product formed by this synthesis should be obtainable from the blood rather than from the urine, as renal abnormality would not confuse the issue. This ideal functional test is not at hand but it appears to be a possibility and all attempts along these lines are worth while and should give information of value. We submit these experiments, which were undertaken as a part of this experimental program to study liver function.

Recognizing the importance of the liver in many synthetic endogenous processes, we felt it would be desirable to extend the study of hippuric acid synthesis under experimental conditions. It has been claimed by some that hippuric acid synthesis is a function of the kidney, by others a part of liver activity—in a word, the question is open to debate. We feel that our experiments furnish new data which will aid in the final solution of this complex physiological equation.

A critical review of the literature touching the influence of benzoic acid and benzoates upon the general metabolism of the experimental animal reveals the fact that the action of this drug is still far from being completely understood. The earlier investigators and particularly Schmiedeburg sought to establish the synthesis of hippuric acid from benzoic acid as a function of kidney activity, using principally what was considered at that time (1876) a very reliable method of transfusion of excised organs. These experiments have stood unquestioned until the last few years, when considerable evidence was adduced to show that the kidney was not alone responsible for hippuric acid production. The work of Kingsbury and Bell (2) with nephrectomized dogs and dogs in which an experimental nephritis had been produced shows little change in the hippuric acid synthesis from the previously normal condition and that in the dog at least the kidney is not the site of the reaction. In rabbits the evidence still appears to be conflicting. On the other hand, the known rôle of the liver in certain processes of metabolism and the influence of benzoic acid on metabolism as shown by our experiments suggests

that the liver might play a definite part in the conjugation of benzoic acid with glycocoll. An analysis of the parenchyma of various organs, after the injection of benzoic acid, shows that there is a higher content of hippuric acid in the liver than in other organs (Kingsbury and Bell). Further evidence that the liver may be involved in the production of hippuric acid is offered by Lackner, Levinson, and Morse (3) who show a definite decrease of the hippuric acid elimination after a liver necrosis, which had been produced by hydrazine sulfate. Dogs were used and benzoate (0.5 to 2.0 gm.) was given by mouth.

The actual mechanism whereby the benzoic acid radicle is neutralized in the body is not understood. The fact that the amount of glycocoll eliminated as hippuric acid is much larger than the amount of glycocoll in the food has led to various explanations which need only to be mentioned in passing. Parker and Lusk (4) suggested the possibility of a glycocoll reserve in the body which can be "washed out" by successive doses of benzoate. The massive breaking down of body proteins is advocated by Ringer (5) and others. The synthesis of glycocoll from simple substances and simple amino-acids may occur (Epstein and Bookman, 6). Lewis maintains that products of metabolism, which might otherwise go to the formation of urea, may be diverted to the production of glycocoll (7) and this hypothesis is confirmed by the work of McCollum and Hoagland (8) who find a decrease in the urea nitrogen elimination of pigs on a carbohydrate diet following the administration of benzoates. Lastly the hypothesis of Umber (9), objected to by Abderhalden (10), that benzoic acid abstracts glycocoll from the globulin molecule of the tissues, thereby converting it to an albumin molecule; and that the action of benzoic acid in the body is merely to change the normal albumin-globulin ratio.

That so many theories exist is evidence that the process is either not understood completely, or that no one explanation is sufficient. Since all hypotheses of internal metabolism are deduced from the study of the urinary nitrogen, and since the conflicting theories advanced are based on conflicting results of urinary analysis, an attempt will be made to show that *different results may be referable to differences in animals used and to differences in dosage.*

Methods.

For the following experiments female dogs were kept in standard metabolism cages and the urine collected at a fixed hour each day. The 24 hour specimen included the cage collection, cage washings, bladder urine, and bladder washings made up to a standard dilution. The dogs were fasted for 3 or 4 days previous to the experiment to allow the urinary nitrogen excretion to reach a constant base line and food was withheld during the entire experiment. Water was supplied in the cage at all times and it was noted that after the injection of sodium benzoate the dogs showed a much greater thirst. The collected urine was subjected to the following analysis: Total urinary nitrogen by the Kjeldahl method; urea and ammonia nitrogen by Marshall's method; free benzoic acid by the method of Raiziss and Dubin (11); hippuric acid by the method of Folin and Flanders (12); all determinations were made in duplicate with suitable controls. A slight modification was made in both the method for the determination of the free benzoic acid and in the method for the determination of the hippuric acid.

Determination of Free Benzoic Acid.—100 cc. of urine are acidified by the addition of 1 cc. of strong nitric acid and 50 gm. of ammonium sulfate are added. To this are added, in a 250 cc. Erlenmeyer flask, 50 cc. of freshly distilled chloroform; the whole is then shaken thoroughly for some time and allowed to stand from 3 to 24 hours. At the end of this time, the chloroform is separated and is transferred into a second Erlenmeyer flask. The residue is washed into the first flask with 25 cc. of fresh chloroform and treated in the same way as the first extraction. The extract is washed by shaking with 100 cc. of saturated common salt solution and allowed to stand over night. On the next day the chloroform is separated in a clean dry funnel and is titrated with a standardized tenth normal alcoholic solution of sodium ethylate (phenolphthalein indicator). It was found convenient to keep the various chloroform fractions separate for the reason that, as was often found, the urine may be free from benzoic acid, a fact which can be noted on the first titration and further extraction with the chloroform rendered unnecessary. In each set of benzoic acid determinations (two daily for each dog) a complete duplicate was carried out with distilled water to insure no acid from the first acidification of the urine being retained in the extraction, and to show that the washing process had been sufficient.

Determination of Hippuric Acid.—100 cc. of urine are rendered alkaline with 10 cc. of 5 per cent sodium hydroxide and are evaporated to dryness on a water bath. The residue is dissolved in 25 cc. of water, and is then decanted into a Kjeldahl flask, and 25 cc. of strong nitric acid are poured into the same flask. A few crystals of copper sulfate are then added and the Kjeldahl flask is fitted with a long reflux condenser; an apparatus may be set up so that four or five determinations may be made at the same time with an extra flask for a control. The material in the flask is now boiled over a small flame for several (3 or 4) hours until the solution is a clear blue. When this stage is reached the condenser is allowed to cool and the inside

is washed down twice with 25 cc. of distilled water. To the material in the flask, the volume of which is now about 100 cc., 50 cc. of freshly distilled chloroform are added and the whole is vigorously agitated for several minutes and is then allowed to stand for several hours. The chloroform is separated in the same way as in the method for the estimation of free benzoic acid and is thus similarly washed and titrated. As in the former method, it is advantageous to keep the extracts separate and to continue with extracting repeatedly until all the benzoic acid has been removed. It is hardly necessary to outline the rationale of the procedure at this time—reference has been made to the sources in which these methods were originally described, where additional and further information will be found.

Raiziss and Dubin (11) advise the use of toluene for the benzoic acid extraction, but in this work chloroform was found more convenient and it was determined experimentally that under the same conditions both solvents extract approximately the same amount of acid in the same time.

It will be seen that the extraction is made from a solution which is very strongly acid, and that if for some reason the chloroform is impure some of the nitric acid will be retained in the chloroform and the titration with the sodium ethylate will be rendered extremely inaccurate. It was found that small quantities of alcohol in the chloroform caused this error, and on this account it was necessary to wash the chloroform repeatedly with salt solution. When it was desired to reutilize chloroform which had been used in a previous extraction, it was rendered strongly alkaline and washed repeatedly. Since the used chloroform contained phenolphthalein, the washing in an alkaline solution was continued until the pink color had disappeared, and the chloroform was then considered ready for the first distillation. The distillation was carried out in the usual way, in a water bath, and the distillate washed by allowing it to stand for 24 hours over saturated salt solution, after which it was distilled a second time. Even the purest commercially obtainable chloroform was treated in this way, and it was found that such treatment was essential to avoid large and unexpected errors.

With the methods outlined above, it was possible in control experiments to recover from an aqueous solution of sodium benzoate an average of 99.8 per cent with a fluctuation of 10 per cent between extremes. The hippuric acid shows a recovery of 92.2 per cent with a fluctuation of 7 per cent between extremes.

It was found, in control experiments, that when bile, blood, or fecal material was added to the urine, and the urine was treated in the usual way for the determination of hippuric acid, that substances of an acid nature appeared in the chloroform, which were capable of neutralizing the sodium ethylate in the titration. The chemical nature of these substances was not satisfactorily determined. If the urine collected under the cage was found

TABLE I.

The Recovery of Hippuric Acid Before and After Anesthesia.

Dog 20-81. Brown spaniel, mongrel, adult.

Date.	Benzoate intravenously.		Hippuric acid.			Urea and ammonia N.	Total N.	Weight.
	Total.	Per pound.						
Jan. 24	Dog isolated and fasting begun.							
1921	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*lbs.*
Jan. 26	0	0	0.470	0.485	—	1.970	2.408	24.5
" 27	0	0	0.425	0.440	—	2.040	2.632	24.0
" 28	0	0	0.510	0.510	—	1.860	2.520	23.5
" 29	0	0	0.542	0.510	—	1.880	2.380	23.3
" 30	4	0.174	4.170	4.070	82.5	2.550	3.690	23.0
" 31	4	0.176	4.850	5.140	102.0	2.340	3.660	22.8
Feb. 1	4	0.178	—	—	—	2.460	3.860	22.5
" 2	0	0	—	—	—	2.460	3.470	21.8
Feb. 2	Chloroform anesthesia—60 minutes.							
Feb. 3	4	0.187	—	—	—	—	5.040	21.5
" 4	4	0.190	5.140	4.400	93.2	—	5.150	21.0
" 5	4	0.200	4.985	4.700	98.6	3.030	4.200	20.0

to be contaminated by blood or bile or if the fecal contamination was in the form of a diarrhea which could not readily be filtered out, the urine sample of that day was discarded for obvious reasons.

Except where specifically stated to the contrary, the benzoate was administered in the form of sodium benzoate, and injected into the jugular vein of the dog in a 5 per cent aqueous solution at the rate of about 20 cc. per minute. In the experiments marked "by mouth" the drug was given through a stomach tube, which was washed out with water.

To determine the effect of chloroform anesthesia upon the synthesis of hippuric acid several experiments were conducted, of which Tables I to III inclusive may be taken as typical examples. It has been shown by Davis and Whipple (13) that after 3 days fasting, 60 minutes of light chloroform anesthesia will produce a central necrosis involving one-half of the liver parenchyma in

TABLE II.

The Recovery of Hippuric Acid in the First 5 Hours after Benzoate Injection Intravenously.

Dog 20–25. Fox terrier, adult.

Date.	Benzoate dose.	Time.	Hippuric acid.			Weight.
1920	*gm.*	*hrs.*	*gm.*	*gm.*	*per cent*	*lbs.*
Oct. 30	3	1–5	1.940	1.750	55.4	22.0
Nov. 3	3	1–5	1.520	1.700	48.0	21.0
" 4	3	1–5	1.820	1.860	55.5	21.5
" 5	3	1–5	1.895	2.070	59.7	21.5
" 17	3	1–5	1.615	1.700	50.0	21.0
" 24	3	1–5	2.340	2.080	66.5	22.0
" 25	3	1–5	2.380	2.520	74.0	22.0
Dec. 1	Chloroform anesthesia—50 minutes.					
Dec. 2	3	1–5	1.265	1.265	38.2	20.5
" 4	3	1–5	1.225	1.270	37.6	19.5
" 6	3	1–5	0.925	0.945	27.9	19.0
Dec. 21	Dog dead from distemper—recovery from chloroform poisoning.					

Diet of bread and milk throughout experiment except on the 2 days preceding chloroform anesthesia.

most cases, usually followed by recovery and liver regeneration. We must refer to the paper of Davis and Whipple (13) for the data establishing the constancy of liver injury under uniform conditions.

From the examination of the percentage amounts of hippuric acid recovered (Table I), it will be seen that the recovery on 2 successive days average 92.2 per cent. After 60 minutes of chloroform anesthesia, the average recovery on 2 successive days is 95.5 per cent per 24 hours. The total recovery, therefore, is

TABLE II-A.

The Recovery of Hippuric Acid in the First 5 Hours after Benzoate Injection. Fatal Liver Injury.

Dog 20-21. Airedale, adult.

Date.	Benzoate dose.	Time.	Hippuric acid.			Weight.
1920	*gm.*	*hrs.*	*gm.*	*gm.*	*per cent*	*lbs*
Nov. 6	3	1–5	2.150	2.00	63.1	21.5
Nov. 24	Chloroform anesthesia—60 minutes.					
Nov. 25	3	1–5	1.100	—	33.1	21.5
Nov. 25	Dog died, acute chloroform poisoning—extreme liver necrosis.					

not influenced by severe liver injury. From this and other experiments of a similar nature, it may be concluded that extensive liver necrosis due to chloroform has very little effect upon the total amount of hippuric acid synthesized during the whole 24 hours.

The possibility suggested itself that there might be a delay in this synthetic reaction. With this in mind, the 24 hour period

TABLE III.

The Recovery of Hippuric Acid from the 5th to the 24th Hour after Benzoate Injection Intravenously.

Dog 20–17. Small black mongrel, adult.

Date.	Benzoate dose.	Time.	Hippuric acid.			Weight.
1920	*gm.*	*hrs.*	*gm.*	*gm.*	*per cent*	*lbs.*
Dec. 23	3	5–24	2.025	2.180	63.70	21.8
" 24	3	5–24	2.010	—	60.60	22.5
" 25	3	5–24	1.980	1.690	55.25	22.0
Dec. 29	Chloroform anesthesia—60 minutes.					
Dec. 31	3	5–24	2.400	(1.850)	64.05	21.0
1921						
Jan. 1	3	5–24	2.480	2.380	73.35	20.0
" 2	3	5–24	2.010	—	60.60	20.0
" 3	3	5–24	2.080	2.090	62.80	19.0

Complete recovery.

was arbitrarily split so that the first collection was made 5 hours after the injection of benzoate and the second collection comprised the specimen of the subsequent 19 hours.

From an examination of Table II, it will be seen that the amount of hippuric acid synthesized and excreted during the first 5 hours after the injection of sodium benzoate averages for a period of 7 days 58.4 per cent of the amount injected; while after 50 minutes of chloroform anesthesia the average for a 3 day period drops to 34.5 per cent. In this experiment, after a period of 2 days fast, chloroform anesthesia was given for 50 minutes. Except for this interval the dog was fed bread and milk. The dog died 20 days later from distemper after recovery from the chloroform poisoning.

Similarly in Table II–A, the recovery during the first 5 hours after the injection of sodium benzoate in the control period averaged 63.1 per cent, while after 60 minutes of chloroform anesthesia it averaged 33.1 per cent. In this experiment, although the bread and milk on which this dog was fed was withheld for only 2 days previous to the chloroform anesthesia, an extreme liver necrosis was produced and the dog died the following day. Histological examination of liver sections shows practically a complete liver cell destruction with only a few small groups of liver cells surviving about the portal tissues.

A liver necrosis was produced in a dog (Table III) by allowing the dog to fast for 2 days and then giving 60 minutes of chloroform anesthesia. Although the lesions thus produced may have comprised one-half or more of the liver parenchyma, the dog recovered from the effect of the intoxication. The effect on the hippuric acid output during the interval between the 5th and the 24th hour is much less marked than is seen in Table II. Before the period of anesthesia in an average of 3 days' determinations, the dog was excreting 59.8 per cent of the dose between the 5th and the 24th hour. After the period of anesthesia, the dog excreted 65.2 per cent during the same time. The dog was fed bread and milk except on the 2 days preceding the chloroform anesthesia.

On the basis of the tabulated and other experiments it would seem proper to conclude that a liver necrosis involving more than one-half of the liver parenchyma produces a *definite delay in the*

hippuric acid conjugation and excretion, so that during the first part of the 24 hours the excretion is relatively less than during the later part of the 24 hours. The total amount of hippuric acid synthesized seems to be little altered if the whole 24 hour period is considered. It will be a matter of dispute whether on the basis of these experiments a further deduction can be made that the synthesis of hippuric acid is a function of the liver. The absence of demonstrable lesions or pathological conditions elsewhere in the body following chloroform anesthesia make it seem probable that the lesions in the liver are the cause of these changes noted in the excretion of the hippuric acid. Control experiments show that dogs injured by chloroform do not present an impairment of renal function to explain the delay in excretion of hippuric acid.

In these experiments it will be seen that the amount of hippuric acid recovered approaches 100 per cent of the amount injected. This, however, was not a constant finding. Dogs of the same weight and under the same conditions, show considerable variation in the amount of hippuric acid eliminated. No correlation could be established between the size of the dog, the size of the dose, and the amount eliminated. In some cases the amount recovered was only 70 per cent of the dose administered, and yet no free benzoic acid was recovered from the urine. These abnormalities deserve further study.

The Rise in Urinary Nitrogen Following Sodium Benzoate Injection.

A number of experiments (nine) were carried out to observe the effect of sodium benzoate in varying doses on the urinary nitrogen, and as the results are very similar, only a few typical examples need be given. Table IV shows the typical rise in urinary nitrogen following the injection of 3.5 gm. of sodium benzoate in a 5 per cent solution into the jugular vein of a 15 pound dog. In this case the total urinary nitrogen excreted averages 1.596 before the injection of the benzoate, and rises to an average of 2.968 gm. after the injection. The greatest part of this rise (excluding the fraction contained in the hippuric acid) is made up of urea, which rises to an average of 2.008 gm. from a normal average of 1.005 gm. The ammonia shows a definite rise over the normal period.

TABLE IV.

The Rise in Ammonia, Urea, and Total Urinary Nitrogen Following Benzoate Intravenous Injection.

Dog 20-96. Small black mongrel, senile.

Date.	Benzoate intravenously.		Hippuric acid.			Urea N.	Ammonia N.	Total N.	Weight.
	Total.	Per pound.							
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*lbs.*
June 24	0	0	0.236	0.286	—	1.085	0.135	1.568	15.00
" 25	0	0	0.498	0.398	—	0.995	0.106	1.652	14.75
" 26	0	0	0.286	—	—	0.935	0.134	1.568	14.50
" 27	3.5	0.185	3.980	3.680	95.2	1.780	0.268	2.688	14.25
			4.070						
June 28	3.5	0.188	4.070	3.750	96.5	1.880	0.204	2.884	14.00
" 29	3.5	0.191	3.640	3.300	94.0	2.350	0.224	3.332	13.75
" 30	0	0	—	—	—	0.558	0.157	1.064	13.50

TABLE V.

The Effect of Gradually Increased Doses of Benzoate on the Urinary Nitrogen.

Dog 20-77. Small fox terrier, brown, adult.

Date.	Benzoate intravenously.		Hippuric acid.			Urea N.	Ammonia N.	Total N.	Weight.
	Total.	Per pound.							
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*lbs.*
Apr. 3	0	0	0.045	—	—	0.739	0.282	1.663	11.00
" 4	0	0	0.110	—	—	0.700	0.590	1.624	10.75
" 5	0	0	0.066	—	—	0.850	0.269	1.512	10.50
" 6	0	0	0.117	—	—	0 810	0.380	1.456	10.00
" 7	0	0	0.088	—	—	0.950	0.095	1.512	10.00
" 8	1.25	0.128	1.380	1.305	94.6	1.120	0.176	1.680	9.75
" 9	1.50	0.158	1.230	1.116	70.0	1.410	0.213	2.016	9.50
" 10	2.00	0.222	1.845	1.775	80.6	1.170	0.240	2.167	9.00
" 11	0	0	0.279	—	—	0.950	0.112	1.512	8.50
" 19	0	0	0.235	—	—	0.660	0.045	1.036	9.50
" 20	0	0	0.252	—	—	0.760	0.067	1.148	9.50
" 21	0	0	0.244	—	—	0.747	0.101	1.232	9.25
" 22	0	0	0.219	—	—	0.803	0.112	1.204	9.25
" 23	2.50	0.278	2.100	1.800	67.5	1.422	0.224	2.142	9.25
" 24	3.00	0.334	2.360	2.130	64.2	1.544	0.246	2.240	9.00
" 25	4.00	0.445	Lethal dose.						

Table V shows the effect of gradually increasing the dose of the sodium benzoate. As the dose is increased from 1.25 to 4 gm., it will be seen that there is a progressive increase in the total urinary nitrogen. The increase in the urea nitrogen corresponds somewhat to the increase in total urinary nitrogen but the relation of the percentage of urea nitrogen to the total urinary nitrogen is very variable. In this experiment doses varying from 0.128

TABLE VI.

Benzoate Given by Mouth and Intravenously.

Dog 20-101. Airedale, pup, weight 20 pounds.

Date.	Benzoate.		Hippuric acid.			Urea N.	Ammonia N.	Total N.	Remarks.
	Total.	Per pound.							
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	
May 8	0	0	0.295	—	—	1.530	0.153	2.380	
" 9	0	0	0.388	—	—	1.395	0.140	2.296	
" 10	0	0	0.338	—	—	1.260	0.134	2.212	
" 11	0	0	—	—	—	1.345	0.112	2.128	
" 12	0	0	0.346	—	—	1.275	0.129	2.184	
" 13	3.0	0.113	3.320	2.610	79.0	1.715	0.258	2.800	Intravenous.
" 14	3.5	0.138	3.880	3.420	88.0	1.760	0.258	2.940	"
" 15	4.0	0.152	4.425	3.830	87.0	1.955	0.244	3.136	"
" 16	0	0	—	—	—	1.155	0.145	2.156	
" 17	0	0	—	—	—	1.382	0.168	2.100	
" 18	3.0	0.113	2.500	2.150	65.0	1.125	0.134	2.016	By mouth.
" 19	3.5	0.135	3.430	3.100	79.8	1.170	0.370	2.268	" "
" 20	4.0	0.154	4.025	3.750	85.5	1.585	0.291	2.660	" "
" 21	0	0	0.512	—	—	1 300	0.179	2.080	

to 0.222 gm. per pound dog fail to produce a definite rise in ammonia, while larger doses seem definitely to increase the ammonia. During the first part of the experiment, the variation in the ammonia is within the physiological variation, but after the 19th, a lower normal base line is maintained which is definitely exceeded after the injection of larger doses. A lethal dose was reached with 0.445 gm. per pound body weight.

To determine whether the method of administration of the drug exerts any influence on its metabolism through possible variations in rate of absorption, injections of the same amount of

sodium benzoate were given intravenously and later by mouth to a dog under as nearly as possible identical conditions. The results of this experiment may be seen in Table VI.

In this experiment the usual marked rise in total urinary nitrogen, urea nitrogen, and ammonia nitrogen follow the intravenous injection. The first dose by mouth was partly vomited, which accounts for the low value of the recovered hippuric acid. On the 19th and 20th, the full dose was retained. It will be seen that the response to the ingestion is very much less marked than

TABLE VII.

Composite Table Showing the Total Urinary Nitrogen with Doses of Sodium Benzoate Varying from 0.079 to 0.445 Gm. per Pound.

Dog No.	Total urinary nitrogen, 3 days' average.	Total urinary nitrogen after injection of benzoate.			Dose of sodium benzoate given per pound.			Dog weight.
		1st day	2nd day	3rd day	1st day	2nd day	3rd day	
					gm.	*gm.*	*gm.*	*lbs.*
20–101	2.306	2.352	2.464	2.372	0.079	0.090	0.102	20.00
20–101	2.240	2.800	2.940	3.136	0.113	0.138	0.152	20.00
20–77	1.553	1.680	2.016	2.167	0.128	0.158	0.222	9.70
18–38	2.760	4.440	3.860	3.330	0.146	0.150	0.150	19.00
20–81	2.485	3.690	3.660	3.860	0.174	0.176	0.178	22.00
20–96	1.596	2.668	2.884	3.332	0.185	0.188	0.191	14.30
20–20	1.344	2.016	2.240	2.240	0.200	0.200	0.200	12.10
18–38	2.760	3.466	3.819	2.979	0.216	0.216	0.220	19.00
20–20	1.222	3.024	3.416	3.248	0.286	0.292	0.298	12.10
20–77	1.155	2.142	2.240	Lethal.	0.278	0.334	0.445	9.70

the response to the intravenous injection, the urea and total urinary nitrogen being almost within the limits of physiological variability and the ammonia alone showing a very slight rise.

Table VII shows that below a dosage of 0.140 gm. of sodium benzoate per pound of body weight, the change in the total urinary nitrogen is not significant. Doses larger than this, however, produce a definite and constant rise in urea, ammonia, and total urinary nitrogen. The greatest responses noted were between the doses of 0.180 and 0.200 gm. per pound. In three dogs with increasing dosage the rise in total urinary nitrogen was progressive to a certain point after which there was a less marked rise; this is shown in Dogs 20–81 and 18–38, and to a lesser degree in Dog

20–20. A sufficient number of observations are not available to determine whether this is a constant finding.

The fasting dog presents a constant base line of nitrogen excretion and the sudden rise following the injection of sodium benzoate must clearly be due to the breaking down of body proteins.

TABLE VIII.

The Rise of Urinary Nitrogen Following Benzoate Injection Prevented by Administration of Dextrin.

Dog 20–20. Fox terrier, adult.

Date.	Dextrin.	Benzoate. Total.	Benzoate. Per pound.	Hippuric acid.			Urea N.	Ammonia N.	Total N.	Weight.
1920	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*lbs.*
May 8	0	0	0	0.210	—	—	0.715	0.075	1.288	13.00
" 9	0	0	0	0.236	—	—	0.890	0.067	1.232	13.00
" 10	0	0	0	0.270	—	—	1.240	0.112	1.624	12.75
" 11	0	0	0	0.442	—	—	0.990	0.177	1.512	12.50
" 12	0	0	0	0.362	—	—	0.920	0.190	1.456	12.50
May 13	0	3.5	0.286	3.510	3.180	88.5	2.260	0.277	3.024	12.25
" 14	0	3.5	0.292	3.720	3.390	96.0	2.390	0.274	3.416	12.00
" 15	0	3.5	0.298	3.540	3.210	90.5	2.240	0.230	3.248	11.75
May 16	0	0	0	0.440	—	—	1.540	0.151	2.172	11.25
June 3	0	0	0	0.218	—	—	0.985	0.089	1.148	12.25
" 4	0	0	0	0.236	—	—	0.975	0.106	1.148	12.00
" 5	0	0	0	0.210	—	—	0.965	0.095	1.262	11.75
" 6	0	0	0	0.270	—	—	0.965	0.078	1.204	11.50
" 7	0	0	0	0.285	—	—	1.020	0.095	1.260	12.00
June 8	30	0	0	0.220	—	—	0.780	0.095	1.064	12.00
" 9	30	0	0	0.255	—	—	0.492	0.218	1.148	12.00
" 10	30	0	0	0.264	—	—	0.589	0.078	0.800	11.75
June 11	30	3.5	0.306	3.440	3.350	86.5	0.962	0.185	1.344	11.50
" 12	30	3.5	0.306	3.640	3.390	87.5	0.995	0.525	1.624	11.50
" 13	30	3.5	0.313	4.040	3.770	97.0	0.975	0.224	1.652	11.25

This breaking down of body proteins was prevented by Epstein and Bookman (6) by the stimulating administration of dextrin, an observation we were able to confirm, as indicated in Table VIII.

A dog fasted for 7 days, during the last 4 of which his normal ammonia, urea, and total urinary nitrogen were determined. A dose of 3.5 gm. of sodium benzoate was injected which practically doubled the urea and total urinary nitrogen. After a period of rest, the same dog again fasted 7 days, during the last 4 of which the same determinations were made and a new base line established. 30 gm. of dextrin were given by mouth, which caused a slight drop in the nitrogen excretion. This was accompanied after 3 days by the same dose of benzoate which had previously caused such a marked rise in nitrogen elimination and little change in the nitrogen output occurred, showing that the dextrin had succeeded in preventing the reaction observed in the control period.

The Effect of Sodium Benzoate Injections on the Blood Serum Proteins.

Several experiments were carried out to determine the effect of benzoate injections on the blood proteins.

Dog 20–101 is a characteristic example.

	Before injection.	After injection.
	per cent	*per cent*
Non-proteins	2.15	2.15
Albumin	3.53	3.71
Globulin	1.69	1.42
Total protein	5.22	5.13

In this experiment, 4 gm. of benzoate were injected and the second determination was made 2 hours after the injection into a 20 pound dog. Determinations were made by the refractometric method of Robertson (14).

If we assume the blood plasma volume of the dog to be 5 per cent of his body weight, the serum globulin 1.7 per cent of his blood plasma volume, and the glycocoll 3.5 per cent of the serum globulin, the dog should have 0.270 gm. of glycocoll combined in the form of serum globulins. On the other hand, 4 gm. of sodium benzoate combine with 1.85 gm. of glycocoll to form hippuric acid, which is actually seven times the amount present in all the blood serum globulins. It is interesting to note that the globulin-albumin ratio remains the same while this demand for glycocoll

is being met. The injection of benzoate does not influence the non-protein nitrogen circulating in the blood.

DISCUSSION.

Our experiments show that a very severe and even fatal liver necrosis due to chloroform may not diminish the total 24 hour amount of synthesized hippuric acid following administration of a unit dose of sodium benzoate. This may suggest that the liver has nothing to do with hippuric acid synthesis, but we believe this conclusion is not justified. Further study shows (Table II) that under conditions of liver injury, there is a *delay in the synthesis and excretion of hippuric acid* in the 5 hour period following the administration of the sodium benzoate. There is no decrease in renal function during this period as indicated by phthalein functional tests. The liver inquiry, therefore, *delays* this reaction which is so prompt in the normal dog. Our belief is that the liver is much concerned in the normal synthesis of hippuric acid in the body but after injury this function may be taken over in large measure by other cell protoplasm. This indicates too that other organs and tissues may take part in this synthetic reaction even under normal conditions.

Our experiments show a definite rise in ammonia, urea, and total urinary nitrogen following the *injection* of sodium benzoate into the circulation of the dog, whenever a certain dosage is exceeded. If these conditions be applicable to other animals, it would seem possible that the failure of some observers to note a rise in urinary nitrogen after administration of benzoate may be explained by the use of small doses. The rise in urea nitrogen is not in agreement with the observations of Ringer, and McCollum and Hoagland, who found that large doses of benzoate decrease the urea nitrogen excreted. It must be remembered, however, that McCollum and Hoagland worked with pigs, and that the caloric requirements of the animals were supplied, whereas in our experiments, the dogs were fasting. In both instances, however, the nitrogen of the urine was wholly endogenous. Our doses per unit body weight exceed those of McCollum and Hoagland and this may explain the differences.

How may we explain this rise in urinary nitrogen following the intravenous injection of sodium benzoate? Perhaps a tentative

explanation may be advanced as follows: When the drug is given intravenously, the demand for conjugation is very acute and lacking the available glycocoll, there is a breakdown of body protein to supply a part of this emergency requirement. We may imagine that certain elements (glycocoll) are removed from the large protein molecule which as a result disintegrates, at least in part. The less acute demand made by administration of the drug by mouth can be met by the usual body mechanism (glycocoll synthesis) unless very large doses are given and there is no emergency breakdown of body protein. The death of the animal we may attribute to this destructive action which may break down body protein in such fashion that certain poisonous split products are formed in sufficient amount to cause fatal intoxication.

The interesting suggestion that glycocoll may be obtained from the globulins of the body finds no support in our experiments. It is known that under certain conditions there may be a rather prompt shift in the albumin-globulin ratio in the blood plasma. Therefore, this suggestion that glycocoll might be furnished in this way by a change of globulin to albumin was worthy of serious consideration. It may be claimed that our experiments do not rule out this possibility but at least we can say that the plasma albumin-globulin ratio does not change after a benzoate injection. If we wish to cling to this hypothesis, we must postulate an effect which is limited to the *tissue globulin-albumin ratio*—this would seem to be indeed a venture into realms of conjecture.

SUMMARY.

The synthesis of hippuric acid in the body following benzoate administration is not prevented by an extensive chloroform liver necrosis. A severe liver injury, however, will cause a distinct *delay* in the synthesis and excretion of hippuric acid. This indicates that the liver normally takes part in this synthesis but that other cell protoplasm of the body may be concerned in this conjugation and may in an emergency take over a greater part of the hippuric acid synthesis. This may apply particularly to the intravenous administration of the benzoate.

Our experiments show distinct increases in ammonia, urea, and total urinary nitrogen wherever dosages of benzoate are given intravenously, exceeding a certain amount per pound body

weight. The question of dosage may explain many discrepancies noted in the literature.

Under certain conditions benzoate injection causes a considerable protein breakdown due probably to the acute need for glycocoll which is taken from the body protein molecule. The suggestion that under emergency conditions the glycocoll may be obtained from the globulins finds no support in our experiments. The serum albumin-globulin ratio is not changed by administration of large doses of benzoate.

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THE BASAL METABOLISM OF UNDERWEIGHT CHILDREN.

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Benedict and Talbot's monograph (1) has given a large amount of material on the basal metabolism of children with which subsequent data can well be compared. It includes not only their own extensive series of observations but also a summary and criticism of earlier work. Their own observations are chiefly on normal children—children in good general health and approximately of the average weight for their height.

Very little has been published on the basal metabolism of underweight children. Some of the infants observed by Benedict and Talbot (2) were underweight, and in general showed a higher basal metabolism per kilo or per square meter than well nourished children. This difference was assigned by the investigators to a smaller proportion of fat and a larger proportion of active protoplasmic tissue in the underweight infants. Murlin and Hoobler (3), who in 1915 published observations on 10 infants varying in age from 2 to 12 months, also found that the underweight infants had a higher metabolism per kilo and per square meter than the normal or fat ones. Murlin and Hoobler compare their own series with one by Howland, and with Benedict and Talbot's; and they find that all but three of the forty-eight underweight and atrophic infants had a higher basal metabolism than the eighteen normal infants. Of the five overweight infants all but one had a lower basal metabolism than the normal ones.

On the other hand, a group of young men who were studied by Benedict and others (4) during a prolonged period of underfeeding showed a basal metabolism progressively lower as their underfeeding continued. It was diminished whether expressed as total calories, calories per kilo, or calories per square meter. The

relative proportion of their active protoplasmic tissue may have gone up as they lost fat, but their "stimulus to cellular activity" had gone down with extensive loss of nitrogen from their bodies. These young men lived on a lower metabolic plane than normal, while the very thin infants lived on a higher metabolic plane. A group of underweight college women recently studied at the University of Chicago,[1] young women in fairly good health supposedly eating according to their appetites, showed a normal metabolism—unlike either the underweight infants or the underfed young men.

Only a few isolated observations on the basal metabolism of underweight older children have been found in the literature—a neurasthenic youth observed by Magnus-Levy (cited by Lusk, 5) who partially starved himself for a year or more and whose basal metabolism per square meter was 33 per cent below normal (similar to Benedict's young men); and the thin brother of Rubner's pair (also cited by Lusk) with a very high metabolism.

How about the basal metabolism of the type of child commonly considered undernourished—the child apparently normal except for marked underweight? Will such a child show the high metabolism of the underweight infant or the low metabolism of the underfed adult?

EXPERIMENTAL PART.

Children Studied.—This paper gives the results of basal metabolism observations on two groups of children, most of them underweight. The first group was made up of fourteen children attending a Health School for underweight children held during the summer of 1920 in the Home Economics Department of the University of Chicago[2]. The second group of 14 was from the University Elementary School.[3]

The standard for underweight employed with both these groups was the commonly accepted one used by various child welfare organizations, and printed in convenient form by the Elizabeth

[1] Unpublished data.

[2] A full report of this experimental school by the director, Lydia J. Roberts, is in preparation.

[3] Our thanks are due to Miss Roberts of the Health School and to Principal Gillett and the teachers in the Elementary School for their helpful cooperation.

McCormick Memorial Fund, Chicago. The figures for the children of our ages in these charts are from Boas, Burk, Bowditch, and Smedley based on measurements of school children.[4] They give standards for age, height, and weight, but the calculations of underweight in this paper are from height-weight relationships only, without age considerations. While it is not believed that by any means the whole question of normal heights and weights of children can be settled by reference to these figures,

TABLE I.

Data for Health School Children.

Name.	Age.		Height.		Weight, nude.		Weight, clothed.	Underweight.	Surface Du Bois.
	yr.	*mo.*	*in.*	*cm.*	*lb.*	*kg.*	*lb.*	*per cent*	*sq. m.*
Cleland.....	12	0	53.7	136.5	66.6	30.3	68.0	4	1.09
John.........	8	1	49.5	125.8	51.0	23.2	51.6	10	0.91
Donald.....	11	5	53.6	136.2	61.5	27.9	63.3	11	1.05
Janis........	11	3	51.5	130.9	53.1	24.2	54.1	11	0.94
Therina.....	10	10	58.0	141.3	74.1	33.7	75.8	12	1.18
Mildred.....	9	11	52.3	132.9	56.7	25.8	57.2	12	0.98
Carl.........	10	10	54.1	137.4	60.7	27.5	62.4	12	1.04
Harold......	10	4	53.9	136.7	60.8	27.6	62.1	12	1.04
Elsie Ss.....	9	7	49.1	124.8	47.9	21.7	48.8	13	0.88
Roy..........	9	3	53.7	136.5	60.6	27.5	62.6	14	1.04
Edmund....	10	11	55.2	142.8	64.0	29.1	64.4	15	1.10
Knox........	9	7	48.1	122.2	46.1	20.9	47.3	16	0.85
Helen.......	9	1	54.7	139.4	59.1	26.8	59.9	18	1.05
Elsie Sa.....	9	3	48.0	122.0	42.0	19.0	43.0	20	0.84

it is true that the relationship serves as the most useful single criterion which we have for selecting "undernourished" children, and it is as such that they are used here. An underweight child is arbitrarily considered one more than 7 per cent below standard.

The data as reported by Miss Roberts for the age, height, weight (with and without clothes), and percentage underweight of the fourteen children from the Health School at the time of entering school are given in Table I. Thirteen of these children were underweight according to the standard used (varying from 10 to 20 per cent below) and one, counted as normal, was 4 per cent under. They ranged in age from 9 to 12 years except one little

[4] See Baldwin, Physical growth and school progress, *U. S. Bureau of Education, Bull. 10,* 1914.

boy of only 8 years. All of the thirteen underweight children had more or less marked circles under the eyes, "winged" shoulders, and other features which characterize the undernourished child. The normal weight boy was normal in other ways. A medical examination of every child, made by Dr. Walter H. O. Hoffman, instructor of internal medicine (pediatrics) in Rush Medical School, revealed no clear symptoms of hyperthyroidism in any of the children. One boy, Harold, was diagnosed as having active tuberculosis of the lungs. Detailed case histories of the children will be given later in Miss Robert's report.

The second group was composed of ten underweight, two overweight, and two approximately normal weight children from the University Elementary School of about the same age as the Health School children. They were observed from March to May, 1921. The ten underweights ranged from 9 to 27 per cent under. The two heavy children were 40 and 12 per cent overweight. These children were weighed nude only, for the metabolism experiment, and to give figures to compare with the standard height-weight chart which is made up from averages of clothed children, 1 pound was added to the nude weight of the girls and 1.5 pounds to the nude weight of the boys, figures obtained by averaging the weight of the clothes of the Health School children. The general data for these children are given in Table II.

Method of Determining Basal Metabolism.—In determining the basal metabolism the Benedict portable respiration apparatus was used with the general procedure described by Blunt and Dye (6). The child came without breakfast, lay quietly on the couch for half an hour, and then was connected with the apparatus by a mouthpiece cut down in size so as to be comfortable. The observations of oxygen consumption were made usually for at least two 10 minute periods with a moment or more rest in between. Occasionally when the child grew tired or restless the time was shortened a little, and in a very few cases in the Health School and more in the Elementary School group, one 10 minute period instead of two was accepted for study. Two stop-watches were used for each period so that the observer had a check on her own reading.

Of course the main problem was to keep the children quiet. Both groups of children, those from the Health School especially, were used to various "tests" and a very friendly relationship

existed between the children and the observer. The Health School group organized a "basal metabolism club"—so called by the children—and especial prestige was attached to belonging to it; also prizes of various kinds were given. During the rest and observation periods the children were read aloud to, and so helped to keep quiet and relaxed. A simple marking device was used to keep count of any movements the child might make in each one of the 10 minutes, a − being recorded if no observ-

TABLE II.

Data for Elementary School Children.

Name.	Age.		Height.		Weight nude.		Weight, clothed.	Weight variation.	Surface Du Bois.
	yr.	*mo.*	*in.*	*cm.*	*lb.*	*kg.*	*lb.*	*per cent*	*sq. m.*
Charles.....	10	6	61.2	154.4	138.6	63.0	140.1	+40	1.60
Margaret...	10	1	55.2	138.0	83.0	37.7	84.0	+12	1.18
Robert......	10	0	49.7	124.4	55.0	25.0	56.5	− 3	0.94
Jean........	10	2	52.9	132.3	62.5	28.4	63.5	− 5	1.02
Fay.........	7	7	46.3	115.9	44.0	20.0	45.0	− 9	0.80
John........	7	11	53.2	133.2	60.3	27.4	61.8	−11	1.00
Caroline....	8	3	50.2	125.5	50.8	23.1	51.8	−12	0.90
Edward.....	11	11	60.4	151.0	84.3	38.3	85.8	−12	1.28
Janice......	8	8	53.4	133.5	57.6	26.2	58.6	−14	0.98
Homer......	12	0	59.4	148.5	76.8	34.9	78.3	−16	1.20
Paul........	11	4	57.3	143.3	67.3	30.6	68.8	−18	1.12
Elizabeth...	8	7	55.4	138.5	58.3	26.5	59.3	−22	1.01
Helen.......	11	11	61.2	153.0	78.5	35.7	79.5	−23	1.18
Bryant.....	9	11	56.7	141.6	58.3	26.5	59.8	−27	1.04

able motion was made, a single + for a very slight movement of the hand or arm, several + signs for a larger movement. More than a very few + signs caused the observation to be discarded.

As a standard, the day's duplicates of oxygen consumed per minute were considered satisfactory only when they agreed within 10 cc. of oxygen and in reality the agreement was usually very much closer than this. For example, the duplicates for Elsie Ss., the Health School child with the most markedly abnormal metabolism were 180 and 181 cc. of oxygen per minute the first day observed, 180 and 170 cc. a month later, and 165 and 168 cc. still later. With a few of the more difficult children several efforts had to be made before a satisfactory determination was obtained and a very few children who were attempted never were sufficiently

quiet to complete the experiment. Most, however, behaved surprisingly well, especially after the strangeness of the first time was past.

Eleven of the Health School children were observed successfully on 3 or more different days; one boy, Roy, only once because he left school and another, Edmund, only once because it was only toward the end of the summer, after he had been taught how to lie still during the school rest periods that he succeeded in doing the experiment successfully. This training in lying quiet which was enforced upon all the Health School children by their daily 1 hour rest period was of great assistance in the metabolism work. In the Elementary School group three children were observed on 3 days and the rest on 2 days.

DISCUSSION.

The results of the metabolism observations are given in Tables III and IV. The observations, calculated for 24 hours, are expressed in the usual three ways—total calories, calories per kilo, and calories per square meter. Figures are also included in these tables for children of the same weights as ours, read from the smoothed curves given by Benedict and Talbot (1) to represent the trend or roughly the average of their metabolism observation. Lastly, there is given the variation of the metabolism of our children from that of Benedict and Talbot's.

Health School Children.—For the Health School group it may be noted that the metabolism of all thirteen of our underweight children is markedly higher than the standard and that of the normal boy close to it. Elsie Ss. is the most extreme case of this excessively high metabolism. Though not the most underweight (only 13 per cent), she was one of the children the most below par in general frailty and nervousness. She produced heat (basal) at the rate of 1,188 calories per day, while the standard child of the same weight produces 860 calories or 39 per cent less. Calculated per kilo, her heat production is 53.1 calories and per square meter 1,350 calories, or 39 and 41 per cent higher than the standard curves. She is not only markedly higher than her point on the curve but much higher than even the highest of the several different children of her weight from whose observations the curve was drawn. That is, every kilo of her body seems to be living at a higher rate than that of the normal child.

This little girl, while showing most markedly the high metabolism, indicates the tendency of the rest. Twelve of the thirteen underweight children in the Health School have a basal metabolism 11 per cent or more higher than the standard curve, as shown by all three ways of calculating, and seven a metablism 20 per cent or more higher. Also, like Elsie Ss., all but one (Carl) of the thirteen children show a higher metabolism not only than the average curve, but than any of the children of similar weight making the curve. The average excesses above the curve for all thirteen Health School children are 22, 25, and 24 per cent; that is, the basal metabolism averages roughly a quarter higher than that of the average child of the same weight. There is no close relationship between the amount underweight of any child and the amount of excess metabolism.

Elementary School Children.—Among the Elementary School group (Table IV) the connection between underweight and high metabolism is not quite so marked as in the Health School, but is still noteworthy. Metabolism not far from normal is shown by the two overweights, one of the normals, and two of the moderate underweights. Moderately excessive metabolism (over 10 per cent and under 20 per cent excess) was observed in five of the underweights, and high excess (20 per cent or over) in the other three. The average excesses for the ten underweight children over Benedict's of the same weight are 14 per cent for the total calories, 16 per cent for the calories per kilo, and 18 per cent for the calories per square meter. These excesses, while not so high, are closely in line with the results with the other group. However, among the Elementary School subjects there was one boy, Robert, of approximately normal weight and yet with very high metabolism. Robert was a difficult subject, probably the most difficult included in this paper, so that it is possible that in spite of fairly close agreement between his duplicate determinations, a certain amount of bodily tenseness during the observations may account for part of his excess. An interesting point about several of the children with very high metabolism is that they are what the school principal calls "problem cases"—children peculiarly difficult to manage in the school.

Comparison of Our Children with the Standard for Same Surface and Same Age.—There are two other sets of comparisons which may well be made with Benedict and Talbot's numerous curves—

TABLE III.

Heat Production, Health School Children.

Name.	Underweight.	Date of metabolism determination.	Nude weight, day of determination.	Total calories.			Calories per kilo.			Calories per sq. m.		
				Observed.	Benedict.	Excess.	Observed.	Benedict.	Excess.	Observed.	Benedict.	Excess.
	per cent	*1920*	*kg.*			*per cent*			*per cent*			*per cent*
Cleland.	4	June 27	30.3	1,126								
		July 15	30.9	1,162								
		" 23	31.4	1,075								
		Aug. 6	31.4	1,254								
		Average.	31.0	1,154	1,150	0	37.2	35.0	6	1,059	985	8
John.	10	June 28	23.2	1,104	950	15	47.6	40.0	19	1,213	1,025	18
Donald.	11	June 22	27.6	1,287								
		July 28	28.5	1,293								
		Aug. 9	29.2	1,391								
		Average.	28.4	1,324	1,075	23	46.6	36.5	28	1,261	1,000	26
Janis.	11	June 26	24.1	988								
		Aug. 13	24.1	1,193								
		" 19	24.1	999								
		Average.	24.1	1,060	900	18	43.9	37.0	18	1,128	950	19
Therina.	12	June 24	33.4	1,281								
		Aug. 4	34.1	1,319								
		" 18	35.2	1,210								
		Average.	34.2	1,270	1,140	11	37.1	33.5	11	1,076	935	15

Mildred.	12	June 26	26.4	1,138								
		Aug. 13	26.9	1,137								
		" 18	27.3	1,267								
		Average.	26.9	1,181	970	22	43.9	35.5	24	1,205	940	28
Carl.	12	July 13	27.1	1,073								
		Aug. 12	28.5	1,115								
		" 17	28.8	1,246								
		Average.	28.1	1,145	1,070	7	40.7	37.0	10	1,100	1,000	10
Harold.	12	Aug. 4	27.9	1,201								
		" 17	28.8	1,289								
		Average.	27.8	1,245	1,060	18	44.8	37.0	21	1,198	1,005	19
Elsie Ss.	13	June 25	21.7	1,260								
		" 27	22.4	1,183								
		Aug. 9	23.1	1,120								
		Average.	22.4	1,188	860	39	53.1	38.0	39	1,350	955	41
Roy.	14	June 30	26.7	1,381	1,040	33	51.7	31.5	38	1,328	1,010	31
Edmund.	15	Aug. 26	30.8	1,248	1,070	17	40.5	35.0	16	1,135	985	15

TABLE III—*Concluded.*

Name.	Underweight.	Date of metabolism determination.	Nude weight, day of determination.	Total calories.			Calories per kilo.			Calories per sq. m.		
				Observed.	Benedict.	Excess.	Observed.	Benedict.	Excess.	Observed.	Benedict.	Excess.
	per cent	*1920*	*kg.*			*per cent*			*per cent*			*per cent*
Knox.	16	July 9	20.9	1,216								
		" 14	21.4	1,072								
		" 30	21.3	1,164								
		Average.	21.2	1,151	900	27	54.3	41.5	31	1,354	1,035	31
Helen.	18	June 23	26.9	1,224								
		July 26	26.7	1,224								
		Aug. 6	28.1	1,292								
		Average.	27.2	1,247	980	27	45.8	35.5	29	1,187	940	26
Elsie Sa.	20	June 19	19.0	1,030								
		" 24	19.1	1,071								
		Aug. 3	20.1	1,023								
		Average.	19.4	1,041	780	33	54.5	40.5	35	1,237	970	30

with their children of the same body surface as ours, and with children of the same ages as ours. In Table V we give the percentage variations resulting from these two comparisons for the Health School children as well as from the weight comparisons repeated from Table III. Our children as compared with Benedict and Talbot's children of the same surface show in most cases as would be expected, excesses closely like those for children of the same weight—on the average the total calories are 21 per cent higher and the calories per square meter 24 per cent higher.

The age comparison brings out somewhat different points. Three of the Health School children were considerably taller than the average height for their age; Therina 4.5 inches, Roy 3.3 inches, and Helen 4.9 inches, and, therefore, the total heat production expected from them would be that for an older child; or, to express the same thing differently, they would be expected to show an excess when compared with children of their own age greater than the excess when compared with children of their own weight or surface. This is true for Therina and Helen, but not for Roy. On the other hand, three of the children were markedly *below* the average height for their age; Janis 2.8 inches, Knox 2.0 inches, and Elsie Sa. 2.1 inches. These children would be expected to show a *lower* excess in total calories when compared with children of their own age than by any other comparisons, which is markedly the case with all three.

In making all these comparisons it must be remembered that Benedict and Talbot expressly state that their curves do not represent mathematical averages, merely show the trend of their observations, and that, of course, considerable variation in metabolism must be expected from child to child. No such thing as an accurate "standard" is presented by them. Our figures would, of course, have little significance if they merely showed irregular variations from the curves. It is the marked excess over Benedict and Talbot's observations, occurring almost without exception in the underweight children that makes our results significant.

From all points of view it seems safe to draw the conclusion that all of the thirteen underweight children from the Health School were metabolizing at an abnormally high rate, and all but two of the ten underweights from the Elementary School.

TABLE IV.

Heat Production, Elementary School Children.

Name.	Weight variation.	Date.	Nude weight.	Total calories.			Calories per kilo.			Calories per sq. m.		
				Observed.	Benedict.	Variation.	Observed.	Benedict.	Variation.	Observed.	Benedict.	Variation.
	per cent	*1921*	*kg.*			*per cent*			*per cent*			*per cent*
Charles.	+40	Apr. 5	63.0	1,728								
		" 6		1,721								
		" 7		1,700								
		Average.		1,716	1,634*	+ 5	27.2	25.9*	+ 5	1,073	1,021*	+ 5
Margaret.	+12	Mar. 29	37.7	1,155								
		" 31		1,218								
		Average.		1,186	1,225	− 3	31.5	33.3	− 5	1,005	925	+ 7
Robert.	− 3	Apr. 4	25.0	1,200								
		" 5		1,145								
		May 27		1,230								
		Average.		1,191	995	+20	47.7	38.7	+23	1,267	1,015	+25
Jean.	− 5	Mar. 25	28.4	1,047								
		" 26		1,042								
		Average.		1,045	1,000	+ 5	36.9	35.0	+ 5	1,002	940	+ 7
Fay.	−9	May 25	20.0	1,003								
		" 26		990								
		Average.		996	800	+24	49.8	40.1	+24	1,245	965	+29

John.	−11	Apr 12	27.4	1,115								
		" 13		1,070								
		Average.		1,092	1,060	+ 3	39.9	37.3	+ 7	1,092	1,010	+ 8
Caroline.	−12	May 10	23.1	1,082								
		" 11		1,000								
		Average.		1,041	875	+19	44.9	38.0	+18	1,157	960	+21
Edward.	−12	Mar. 3	38.3	1,358								
		" 4		1,312								
		Average.		1,335	1,285	+ 4	34.8	32.0	+ 9	1,043	960	+ 9
Janice.	−14	Mar. 4	26.2	1,061								
		" 18		1,063								
		Average.		1,062	950	+12	40.4	36.0	+12	1,084	945	+15
Homer.	−16	Apr. 26	34.9	1,342								
		" 27		1,326								
		Average.		1,334	1,225	+ 9	38.1	33.7	+13	1,112	970	+15
Paul.	−18	Apr. 26	30.6	1,490								
		" 27		1,488								
		Average.		1,489	1,130	+34	48.6	35.2	+38	1,329	990	+34
Elizabeth.	−22	Mar. 10	26.5	1,060								
		" 12		1,075								
		Average.		1,063	955	+11	40.1	35.9	+12	1,052	945	+11

* Calculated from the tables in Harris, J. A., and Benedict, F. G., *Carnegie Inst. Washington, Pub. 279*, 1919.

TABLE IV—*Concluded.*

Name.	Weight variation.	Date	Nude weight.	Total calories.			Calories per kilo.			Calories per sq. m.		
				Observed.	Benedict.	Variation.	Observed.	Benedict.	Variation.	Observed.	Benedict.	Variation.
	per cent	*1921*	*kg.*			*per cent*			*per cent*			*per cent*
Helen.	−23	Mar. 27	35.7	1,330								
		Apr. 27		1,402								
		" 29		1,330								
		Average.		1,354	1,180	+15	38.0	33.5	+13	1,147	930	+23
Bryant.	−27	Apr. 12	26.5	1,195								
		" 19		1,124								
		Average.		1,160	1,035	+11	43.7	38.0	+13	1,115	1,010	+10

Day by Day Variation in Metabolism.—The discussion above has been based upon the average of the determinations for several different days for most of the children. Two questions arise in connection with this procedure: Were the results for the different days in close agreement; and was there any regular change in the children's metabolism in the Health School as the summer progressed and the children improved in general condition?

TABLE V.

Variation in Heat Production from Benedict and Talbot's Figures, Health School Children.

Name.	Underweight.	Compared with children of same weight.			Compared with children of same surface.		Height variation, McCormick charts.	Compared with children of the same age.		
		Total calories.	Calories per kilo.	Calories per sq. m.	Total calories.	Calories per sq. m.		Total calories.	Calories per kilo.	Calories per sq. m.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Cleland	4	0	6	8	6	8	—0.7	—4	11	8
John	10	15	19	18	16	19	+0.6	15	21	17
Donald	11	23	28	26	25	27	—0.9	15	35	27
Janis	11	18	18	19	15	19	—2.8	5	27	18
Therina	12	11	11	15	12	17	+4.5	27	6	12
Mildred	12	22	24	28	23	27	+0.8	22	22	25
Carl	12	7	10	10	9	11	+0.8	12	15	10
Harold	12	18	21	19	19	21	+1.5	13	24	18
Elsie Ss	13	39	39	41	37	41	—0.7	25	48	40
Roy	14	33	38	31	31	34	+3.3	34	36	30
Edmund	15	17	16	15	13	16	+1.7	10	14	14
Knox	16	27	31	31	28	31	—2.0	10	45	33
Helen	18	27	29	26	23	26	+4.9	34	24	22
Elsie Sa	20	33	35	30	24	27	—2.1	11	49	27
Average of all but Cleland		22	25	24	21	24		18	28	

With the Elementary School, where most duplicates were obtained on consecutive or almost consecutive days, the agreement between the different days was decidedly close; for ten of the fourteen children the maximum difference was 5 per cent or less, and for the other four it was between 6 and 8 per cent.

In the Health School the agreement between different days is not so close, but also the intervals covered were very much longer, and the number of observations on most children 3 or 4 instead of 2 or 3. Only two of the children show as little as 5 per cent difference between duplicates, one shows as high as 20 per cent, and the average of all differences is 11 per cent. This is of interest in comparison with the rather wide day by day variation found by Blunt and Dye (6) for their normal women. There is no regular change in the children's metabolism as the summer progressed, in spite of general improvement in health. Apparently the time was not sufficiently long and the gains in weight sufficiently great for their metabolism to go down to that of normal children.

SUMMARY.

Metabolism determinations made on twenty-eight children, mostly underweight, showed that the basal metabolism of underweight children tends to be higher than that of the normal child. The excess metabolism was in some cases as high as 40 per cent above that read from curves given by Benedict and Talbot, and in most cases the metabolism was not only higher than the curve but higher than the highest observation of the child of the same weight from which the curve was drawn. The average percentage excesses for the underweight children in the Health School compared with Benedict and Talbot's of the same weight were 22 for the total calories, 25 for the calories per kilo, and 24 for the calories per square meter. In the Elementary School the corresponding excesses were somewhat less—14, 16, and 18 per cent. No close relation was observed between the percentage underweight and the excess metabolism.

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RELATION BETWEEN THE CHLORIDE CONTENT OF THE BLOOD AND ITS VOLUME PER CENT OF CELLS.

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Analyses have shown that plasma contains a higher chloride percentage than is generally found in whole blood (McLean and Van Slyke, Van Slyke and Donleavy, McLean, and Austin and Van Slyke). The experiments reported in this paper were undertaken to determine the relation between the chloride content of the blood and its cell volume percentage. The examination of the blood was based on a micro determination of the chlorides and a determination of the volume of the blood cells.

The following technique was used. 0.5 cc. of 3 per cent sodium citrate solution is measured off in each of two graduated, cylindrical 5 cc. centrifuge tubes (Oluf Thomsen's tubes for the blood platelet count). By venous puncture approximately 4.5 cc. of blood are added to each of the tubes, and the blood and citrate mixed by corking and inverting. Any possible variation from the exact measure is read off on the scale graduated to 0.1 cc.

One of the tubes is reserved for the determination of the volume per cent of the blood cells, while the other is used for the microchloride determination on citrated blood and citrated plasma in the manner described below.[1]

The chloride content of the blood is calculated as sodium chloride and is given in per cent of 100 gravimetric parts of whole blood or plasma.

[1] In regard to the exchange of chlorides between corpuscles and plasma due to differences in the CO_2 tension (Fridericia), our constant results tend to show that we must have studied blood with a nearly constant CO_2 tension. The tubes were corked immediately after the taking of the blood and were only opened for a moment to withdraw the blood or plasma for analysis.

The micro determination of Ivar Bang has been used. This method is based on extraction of about 100 mg. of blood absorbed on filter paper with 92 per cent alcohol for 5 hours. The alcoholic extract plus an equal volume of alcohol, used for washing out the test-tube containing the blood-imbibed paper, are titrated with a 0.01 N $AgNO_3$ solution. The chloride values obtained by this technique are somewhat lower than those by the former methods. Bang found that the extraction of the chlorides is complete after 5 hours. However, it seems that the process tends toward a balance of distribution between the coagulum and the alcohol. Thus the extraction cannot be complete with one portion of alcohol and even a second extraction does not absorb all the chlorides from the blood, though the succeeding extractions after the second will contain only barely measurable quantities of chloride.

In our preparations for this work 400 specimens of blood, serum, ascitic fluid, edema fluid, pleural exudate, and cerebrospinal fluid were examined by successive extractions.

The average of the chloride percentage calculated from the first extraction was 0.476 per cent; the second, 0.047 per cent; the third, 0.007 per cent; the fourth, 0.0003 per cent; the fifth, 0; the sixth,-0.0005 per cent; and the seventh, 0.

For practical purposes it will be sufficient to extract twice with alcohol, since the second portion plus the alcohol used for washing out the tube after this extraction contains about 10 per cent of the amount determined by the first extraction and since the third and following extractions will contain only unimportant traces of chloride.

The results in the present series of observations confirmed this inasmuch as the average content of chlorides found in the second alcoholic extraction was 8.7 per cent of the average amount found in the first.

This phenomenon is not unknown from other similar processes and is a natural consequence of the laws of balance for the adsorptive distribution between the chlorides extracted in the alcohol and the amounts left in the coagulum.

Bang titrated with a 0.01 N $AgNO_3$ solution. We have used the solution of Mohr diluted 20 times; *i.e.*, 1.4521 gm. of $AgNO_3$ and 1,000 cc. of water. The titration has taken place in diffuse

daylight with a micro-burette and potassium chromate as indicator.

A standard color for the titration has been made in the manner described below, which is a very important point, if exact determinations are to be made.

The procedure, in brief, is as follows:

To a test-tube, G_1, containing 15 cc. of the alcoholic extract, add, drop by drop, enough (n cc.) $AgNO_3$ solution to give a distinct brownish color. The glass is kept in the dark for 15 minutes, which makes it slightly less colored.

To another test-tube, G_2, with the same amount of alcohol, add n cc. of $AgNO_3$ solution. A small addition of silver nitrate solution to G_1 makes the color of both mixtures identical. This tint does not alter appreciably during the titration if the light is not too strong; in which case it becomes somewhat darker. The value n is subtracted from the total number of cc. added to G_1 before the calculation. Each cc. of the $AgNO_3$ solution used in excess of n means 0.5 mg. of sodium chloride. The titer of the $AgNO_3$ solution was tried before titration with a measured quantity of a standard solution of NaCl.

The duration of the first extraction with alcohol has always been 24 hours, and the second at least 24 hours. The chloride values given are the average of two or three determinations which very rarely diverged appreciably.

The calculation from the percentage in citrated blood and citrated plasma to uncitrated blood and uncitrated plasma has been made as stated under the description of the determination of the cell volume. *The contents of chloride found in citrated blood and plasma must be reduced to the contents in uncitrated blood and plasma.*

The reduction of results from citrated blood to those for uncitrated blood is simple. If 0.5 cc. of citrate is mixed with 4.5 cc. of blood, then 100 parts of citrated blood will contain 90 parts of uncitrated blood. If, on the other hand, the same quantity of citrate is mixed with 4.7 cc. of blood, then the corresponding volumes are 100 parts of citrated blood and 90.4 parts of uncitrated blood.

The correction necessary to reduce citrated plasma to uncitrated plasma is a little more complicated, since one must know: (*a*)

the osmotic properties of the citrate solution; (*b*) the quantities of citrate and blood mixed; and (*c*) the volume per cent of the blood cells.

It is evident that if the citrate solution is not isotonic, water or salts will enter or leave the corpuscles, thus altering the concentration of the citrated plasma. We have chosen the 3 per cent citrate solution because it does not alter the cell volume, as will be seen from cell volume determinations (Table I) made on defibrinated blood.

Table I shows clearly that the admixture of a 3 per cent sodium citrate solution to the blood in the ratio 1:9 does not alter the cell volume.

TABLE I.

Cell volume of pure defibrinated blood.	Cell volume of 4.5 cc. of defibrinated blood + 0.5 cc. of 3 per cent citrate.*
per cent	*per cent*
40	40
43	43
54†	55
71†	70
68†	68

* Values corrected for admixture of citrate.

† The last three specimens of defibrinated blood were concentrated by drawing off some serum in order to exaggerate and make visible any slight variation.

On the other hand, a determination of the cell volume on fourteen double specimens, of which one was mixed with a 10 per cent sodium citrate solution showed that in this way there is a considerable shrinkage amounting to 16 per cent of the shrunken cell volume in the citrated blood.

The technique used for the determination of the cell volume was very simple. The tube set aside for this purpose was centrifuged for 90 minutes at 3,000 revolutions a minute. The cell precipitate, including the white cell layer, was read off and the cell volume per 100 cc. of uncitrated blood, volume per cent, calculated as shown in the following example:

4.5 cc. of blood are mixed with 0.5 cc. of 3 per cent citrate and give a cell precipitate of 1.80 cc. The cell volume percentage is then $\frac{1,800}{45} = 40$ volumes per cent.

Of course it has to be proved that centrifuging in the way described does give the absolute cell volume. If this is the case three precautions must by observed: (1) The centrifuge must be kept at an even high speed and slow down *very* gradually; (2) the tubes must fit exactly into the centrifuge so that they do not wobble; and (3) in no case must the time of the centrifuging be cut down, even though 90 minutes allows a rather wide margin.

In order to test our technique, it has been compared with a determination of the cell volume by the hematocrit method, the capillary tubes of the hematocrit being filled from the tube

TABLE II.

No.	Cell volume.		Difference.
	Hematocrit.	Centrifuge.	
	Average of two determinations.	One determination.	
	per cent	*per cent*	*per cent*
1	43	43	0
2	30	31	+1
3	39.5	40	+0.5
4	43.5	43	−0.5
5	37	38	+1
6	28	29	+1
7	64.5	66	+1.5
8	63.5	64	+0.5
9	44.5	45	+0.5
10	33.5	33	−0.5
11	31	31	0
Average.......	41.5	42	

of citrated blood. The volume of the blood absorbed in this way is inconsiderable and cannot be read off on the scale.

The hematocrit employed made about 9,000 revolutions a minute and the blood was centrifuged with an interval every 15th minute, stopping when the blood column had been constant in length thrice and transparent throughout. The results are given in Table II.

The variation between the results is inconsiderable, even if the hematocrit results are slightly lower. Even the largest variation, in a case of polycythemia, No. 7, will not materially affect

our calculations. The manner in which the chloride content of citrated plasma is reduced to that for uncitrated plasma is indicated in the following example:

0.5 cc. of citrate is mixed with 4.5 cc. of blood and gives on centrifuging a cell precipitate of 1.80 cc. The cell volume is then 40 per cent.

If the other specimen contains the same proportions of citrate and blood, then 100 volumes of citrated blood contain 64 volumes of citrated plasma, or 54 volumes of uncitrated plasma. Thus 100 volumes of citrated plasma contain 84.4 volumes of uncitrated plasma.

If the chloride percentage in citrated plasma is found to be 0.48 then the real plasma percentage is 0.57, as the citrate solution used proved to be absolutely free from chloride contamination.

In giving the results of our determinations of the chloride percentage in plasma (and blood) we have divided the cases examined into groups.

Cases with Normal Blood.—As such, have been tabulated cases in which the cell volume varied between 40 and 50 per cent of the blood,[2] and where the individual examined did not show symptoms which might influence the composition of the blood. The material consists of fifteen cases (five men and ten women) showing a cell volume within the above limits. The chloride percentage in plasma calculated as sodium chloride varied between 0.59 and 0.63 per cent, the mean value being 0.61 per cent. *We may in this connection mention that this value, 0.61 per cent, is the same that we have found as an average result in all the thirty specimens of edema fluid which we have examined.*

Table III, as well as the following tables, gives the name, sex, age, diagnosis, true cell volume, and sodium chloride per cent in the blood and in the plasma. The percentage in citrated plasma or blood is not given in these tables.

The variation in the chloride content of the plasma is so small that it may be considered constant. The plasma percentage of chlorides is the same in both sexes, though men on the average have larger cell volumes (and hemoglobin values).

Diseases in Which the Salt Metabolism Might Possibly Be Affected.—In the four cases belonging to this group the cell

[2] 40 per cent is not the lowest normal cell volume, since down to 36 per cent may be found in perfectly sound women. For our purposes, which deal only with the purely mechanical proportions between plasma and the cell volume, this is of no importance.

TABLE III.

Normal Cases.

No.	Name.	Sex.	Age.	Diagnosis.	Cell volume.	NaCl in blood.	NaCl in plasma.
			yrs.		*per cent*	*per cent*	*per cent*
1	A. N.	Male.	35	Normal.	50	0.46	0.62
2	J. N.	"	24	Sequela poliomyelitis.	45	0.45	0.59
3	J. M.	"	21	Normal.	44	0.47	0.63
4	C. S.	"	36	Neurasthenia.	42	0.47	0.60
5	U. P.	"	47	Disseminated sclerosis.	41	0.48	0.62
6	C. C.	Female.	42	Encephalitis.	48	0.47	0.60
7	F.	"	33	Normal.	44	0.50	0.61
8	R. N.	"	17	Struma.	43	0.50	0.59
9	O. E.	"	35	Goiter.	41	0.48	0.62
10	G. F.	"	19	Pregnant.	40	0.48	0.60
11	A. W.	"	31	Normal.	40	0.49	0.63
12	N. R.	"	48	"	40	0.53	0.62
13	M. T.	"	44	Sciatica.	40	0.47	0.60
14	L. A.	"	57	Encephalitis.	40	0.47	0.59
15	J. C.	"	17	Rheumatism.	40	0.49	0.62
Average					42.5	0.481	0.609

TABLE IV.

Cases with Possibilities of Disturbed Salt Metabolism.

No.	Name.	Sex.	Age.	Diagnosis.	Date.	Cell volume.	NaCl in blood.	NaCl in plasma.	Remarks.
			yrs.			*per cent*	*per cent*	*per cent*	
1	E. P.	Male.	15	Edema fugax (Quincke).	Apr. 22	48	0.46	0.61	Before attack.
					May 5	47	0.48	0.61	During attack.
2	B. J.	Female.	54	Pneumonia.	June 7	42	0.46	0.58	
3	C. C.	Male.	17	Albuminuria.	" 8	40	0.45	0.58	
4	F. J.	"	60	Chronic nephritis.	Apr. 23	40	0.50	0.62	
Average						43.5	0.470	0.600	

volume varied within the limits arbitrarily decided to be normal (40 to 50 per cent).

The sodium chloride percentage in the plasma varies between 0.58 and 0.62 per cent. Even if the value 0.58 is a little lower than any found in our normal cases, the variation is so small that it may be considered as normal.

Polycythemia.—By a coincidence, the cell volume was the same in the three cases observed, being 64 per cent.

In these cases the sodium chloride percentage in the plasma varied between 0.57 and 0.62 per cent, being nearly the same as in the cases already mentioned, though the amount of plasma per unit volume of blood was considerably reduced in comparison with the normal cases.

TABLE V.

Polycythemia.

No.	Name.	Sex.	Age.	Diagnosis.	Date.	Cell volume.	NaCl in blood.	NaCl in plasma.
			yrs.			*per cent*	*per cent*	*per cent*
1	M. T.	Male.	59	Polycythemia.	Apr. 21	64	0.46	0.60
2	J. C.	Female.	44	"	May 3	64	0.42	0.57
3	M. L.	Male.	65	"	" 6	64	0.44	0.62
Average						64	0.440	0.597

Simple Anemia.—As such, were tabulated all cases examined where the cell volume was less than 40 per cent and the color index of the blood less than 1. The leucemias, however, were relegated to a special group.

The examination of the blood in these ten cases of which three have been examined twice, shows a variation of the cell volume between 37 and 13 per cent. Nevertheless, we find in the plasma a sodium chloride percentage between 0.58 and 0.63 that is nearly within the same limits and with the same average, as we have found not only in normal blood but also in polycythemia, the opposite condition of anemia.

There is in these cases no relation between the cell volume and the chloride content of the plasma. The highest plasma-chloride value is found with a cell volume of 30 per cent, the lowest with a cell volume of 37 per cent.

TABLE VI.

Simple Anemia.

No.	Name.	Sex.	Age.	Diagnosis.	Date.	Cell volume.	NaCl in blood.	NaCl in plasma.
			yrs.			*per cent*	*per cent*	*per cent*
1	G. V.	Female.	24	Pregnant.	Apr. 17	37	0.50	0.60
2	G. J.	"	8	Splenic anemia.	May 22	37	0.48	0.59
					June 1	35	0.52	0.61
3	J. R.	Female.	30	Goiter.	Apr. 24	37	0.50	0.60
4	J. V.	"	32	Anemia.	June 5	36	0.51	0.61
5	H. J.	Male.	18	Gastric ulcer.	May 29	35	0.48	0.59
					June 9	37	0.48	0.58
6	S. S.	Male.	48	Goiter.	May 27	31	0.52	0.61
7	A. P.	Female.	37	Gastric ulcer.	Apr. 29	30	0.53	0.63
8	L. O.	"	34	Simple anemia.	June 11	27	0.53	0.61
9	J. K.	Male.	56	" "	" 4	24	0.57	0.62
10	N. M.	"	46	" "	Apr. 22	13	0.58	0.60
					May 28	16	0.55	0.59
Average						30.4	0.519	0.603

TABLE VII.

Pernicious Anemia.

No.	Name.	Sex.	Age.	Diagnosis.	Date.	Cell volume.	NaCl in blood.	NaCl in plasma.	Remarks.
			yrs.			*per cent*	*per cent*	*per cent*	
1	J. J.	Female.	28	Pernicious anemia.	May 27	27	0.53	0.63	
2	F. U.	Male.	36	" "	June 10	22	0.57	0.64	More exactly, 0.636.
3	V. N.	Male.	55	Pernicious anemia.	Apr. 27	22	0.56	0.63	
					May 31	10	0.57	0.62	
					June 7	5	0.59	0.63	
4	A. F.	Female.	49	Pernicious anemia.	May 1	18	0.55	0.61	
5	E. P.	Male.	64	" "	June 14	17	0.55	0.62	
6	J. A.	Female.	39	" "	Apr. 27	16	0.54	0.61	
					May 5	13	0.54	0.60	
Average						16.7	0.556	0.621	

Three other specimens with a cell volume of 37 per cent, however, show a chloride percentage of 0.60, 0.59, and 0.60 in the plasma, and in the two cases with the lowest cell volume the chloride percentage in plasma is 0.59 and 0.60.

Pernicious Anemia.—This group comprises six cases, of which one has been examined three times and another twice.

The cell volume in these cases varies between 27 and 5 per cent, so that all cases are pronounced anemias and some even among the most severe. The sodium chloride in plasma varies between 0.60 and 0.636 per cent with an average of 0.62 per cent.

Even if the average values found lie a little higher than those found in the other groups, the variations are too slight to allow any conclusions to be drawn. We do not doubt, however, that

TABLE VIII.

Malignant Tumors.

No.	Name.	Sex.	Age.	Diagnosis.	Date.	Cell volume.	NaCl in blood.	NaCl in plasma.
			yrs.			*per cent*	*per cent*	*per cent*
1	O. P.	Male.	41	Carcinoma oesophagi.	Apr. 20	41	0.49	0.57
2	V. M.	"	62	" hepatis.	June 4	31	0.49	0.57
3	N. J.	"	23	Hodgkin's disease.	Apr. 24	17	0.53	0.58
Average						29.7	0.503	0.573

the plasma in these cases differs from normal plasma or the plasma found in simple anemia, but the difference is too small to permit one to say whether one single specimen comes from a patient with simple or pernicious anemia.

Therefore, we shall, in view of the much larger differences in the chloride percentage of whole blood, consider the chloride percentage of plasma from pernicious anemia as being the same as in the plasma from the other patients.

Malignant Tumors.—Among the patients examined we find two cases of cancer and one case of Hodgkin's disease, all verified post mortem. These three cases, only two of which were anemic, are put in a special group.

Though the cell volume in these cases varies from 41 to 17 per cent, the plasma contains about the same amount of chlorides

in all three cases. The chloride in plasma varies between 0.57 and 0.58 per cent, that is about the lower limit found in the normal cases.

It is of course not permissible to draw any far reaching conclusions from material consisting of only three cases, where the divergence consists in that the mean value is lower than usual.

We shall, as we did in the cases of pernicious anemia, consider these values as lying within the limits 0.57 to 0.64 per cent which we put forward as the boundaries of the normal chloride percentage of the plasma.

Leucemia.—Finally we studied two cases of lymphatic leucemia. Both cases were anemic and the volume determination gives the total volume of red and white cells; the partial volume of the latter, however, was not very considerable.

TABLE IX.

Lymphatic Leucemia.

No.	Name.	Sex.	Age.	Diagnosis	Date.	Hemoglobin.	Cell volume.	NaCl in blood.	NaCl in plasma.
			yrs.			*per cent*	*per cent*	*per cent*	*per cent*
1	J. B.	Male.	68	Lymphatic leucemia.	June 2	71	37	0.51	0.63
2	H. H.	"	63	" "	May 25	52	22	0.55	0.63
					June 11	47	24	0.55	0.62
Average							27.7	0.536	0.627

The cell volume varied between 37 and 22 per cent, but the sodium chloride percentages in plasma were nearly the same in all three determinations. These values, 0.62 to 0.63 per cent, lie rather near the upper limit, but do not pass beyond it.

From Tables III to IX the following conclusions may be drawn.

1. In normal persons we have found a sodium chloride concentration in the plasma which varies but slightly and whose average value may be put at 0.61 per cent, which is the same amount that we have found in edema fluid.

2. In some diseases (angioneurotic edema, pneumonia, albuminuria, and nephritis) where abnormalities of the chloride metabolism take place or might be supposed to take place, we find chloride percentages in the plasma not differing from those found in normal cases.

3. Sodium chloride percentages between 0.57 and 0.63 in the plasma have been found in cases of polycythemia and simple anemia. Even the most extreme forms of these diseases (cell volume 13 to 64 per cent) fall within these boundaries which we consider the boundaries of the normal plasma-chloride concentration.

4. In the six cases of pernicious anemia and the two cases of lymphatic leucemia examined we found a chloride percentage in the plasma within the limits found for the diseases mentioned above. The mean values, however, lie somewhat above those found in the other cases.

5. In three cases of verified malignant neoplasia the sodium chloride percentage in the plasma also were found to lie within the physiological limits, though the mean value was placed near the lower limit.

6. The average of the sodium chloride content in the plasma is 0.61 per cent, but the amounts vary between 0.57 and 0.64 per cent.

The changes in the chloride percentage in the blood will appear from our previous tables. In Table X we have arranged the results according to the cell volumes found. In this way the effects of a change in the cell volume are most clearly demonstrated, and for the sake of comparison the corresponding chloride contents per 100 gm. of plasma are given.

Table X includes all our material, and cell volumes between 64 and 5 per cent are represented. The chlorides in 100 gm. of plasma vary between 0.59 and 0.63 per cent, while the chlorides in 100 gm. of blood vary between 0.44 and 0.59 per cent.

The table emphasizes what we have already shown, that the chloride percentage in the plasma is not influenced in any way by variations in cell volume.

On the other hand, it shows most clearly that the chloride percentage in the total blood increases when the cell volume drops. The variation is rather large, from 0.44 to 0.59 per cent.

That the opposite condition, polycythemia, will show a blood chloride percentage lower than the normal, has to our knowledge never been observed, but appears with absolute certainty from our results.

As our analyses have shown that the plasma percentage of chlorides is nearly constant, while the blood percentage of chlorides will increase when the cell volume decreases, the explanation of this phenomenon must be sought in the fact that the blood

TABLE X.

Cell volume.	NaCl in blood.	NaCl in plasma.
per cent	*per cent*	*per cent*
64–63	0.44	0.60
62–61		
60–59		
58–57		
56–55		
54–53		
52–51		
50–49	0.46	0.62
48–47	0.47	0.61
46–45	0.45	0.59
44–43	0.49	0.61
42–41	0.48	0.60
40–39	0.49	0.61
38–37	0.49	0.60
36–35	0.50	0.60
34–33		
32–31	0.51	0.59
30–29	0.53	0.63
28–27	0.53	0.62
26–25		
24–23	0.56	0.62
22–21	0.56	0.63
20–19		
18–17	0.54	0.60
16–15	0.55	0.60
14–13	0.56	0.60
12–11		
10– 9	0.57	0.62
8– 7		
6– 5	0.59	0.63

corpuscles contain a smaller percentage of chlorides than the plasma.

If the blood is rich in corpuscles, polycythemia, it will be poor in plasma, thus making the total amount of chlorides low, 0.44

per cent; while on the other hand, a blood which is poor in corpuscles, anemia, will contain more plasma, thus causing an increase of the chloride percentage of the whole blood up to 0.59 per cent.

We have not directly analyzed the content of chlorides in the corpuscles, but from our analyses of blood and plasma we may calculate this value in the following way:

If the chloride concentration in the blood is called C_b, the chloride concentration in the plasma C_p, and the chloride concentration in the corpuscles X, then the last value may be calculated from the formula:

$$100 \cdot C_b = C_p \cdot (100 - \text{volume per cent}) + (X \cdot \text{volume per cent})$$

where all values excepting X are known.

If in this equation we introduce the mean values for C_b, C_p, and volume per cent found in Table III of our fifteen normal cases, it will read as follows:

$$100 \cdot 0.481 = 0.609 \cdot (100 - 42.5) + X \cdot 42.5$$
$$X = 0.31 \text{ per cent}$$

The content of chlorides in normal corpuscles then should be about 0.31 per cent.

If in the above equation we introduce in succession the mean values from Tables IV to IX we get in this order the following chloride percentages in the corpuscles: 0.30, disturbed salt metabolism (?); 0.35, polycythemia; 0.33, simple anemia; 0.23, pernicious anemia; 0.30, malignant tumors; and 0.30, leucemia.

The only very pronounced variation in the salt content of the corpuscles is found in the cases of pernicious anemia.

If in the equation used above we call X, C_c (corpuscle-chloride concentration) it will read thus:

$$100 \cdot C_b = C_p \cdot (100 - \text{volume per cent}) + C_c \cdot \text{volume per cent} \quad (1)$$

In this formula one may, according to our experience, call C_p and C_c constant,[3] introducing instead the values 0.61 and 0.31 per cent which gives the formula:

$$C_b = \frac{61 - (0.3 \cdot \text{volume per cent})}{100} \quad (2)$$

It is seen from equation (2) that there is a simple relation between the cell volume and chloride content of the blood and this

[3] Except, however, the cases of pernicious anemia where C_c varies.

relation will persist even if the blood is diluted with edema fluid. One cannot find a higher chloride percentage in hydremia than that which may be calculated from formula (2). The maximal chloride content in the blood will be obtained by putting volume per cent = 0, in which case one finds:

$$C_b = C_p$$

which means that the chloride content of the plasma is the limit.

A chloride content of the blood higher than the plasma percentage could conceivably occur only under two conditions: (1) The blood is diluted with a liquid richer in sodium chloride than edema fluid. Such an occurrence in the body is improbable. (2) The blood corpuscles could store a larger amount of chlorides. We have never found this to be the case, the corpuscle-chloride concentration being lower than the plasma-chloride concentration in all cases observed.

The earlier determinations of the chloride content of whole blood have been a disappointment for the reason that the plasma concentration of the chlorides is nearly constant and the concentration of chlorides in whole blood is a function of the cell volume.

The introduction of the cell volume percentage of the blood in these calculations is of the utmost importance. This value plays an important rôle in the estimation of such substances as are not evenly distributed between plasma and corpuscles. Of course this is even more essential as regards substances which are found only in plasma.

SUMMARY.

1. A 3 per cent (isotonic) citrate solution is used to obtain plasma for analysis.

2. The cell volume percentage in the blood is determined and shown to be a factor to be considered when the concentration of a substance is determined on whole blood.

3. In 52 cases of various types we find that the content of sodium chloride in the plasma is nearly constant, about 0.61 per cent.

4. The corresponding chloride determinations on whole blood show that these values vary greatly. It is shown that the chloride

percentage in blood increases when the cell volume percentage (and hemoglobin) drops, and *vice versa;* these changes following certain laws, which are formulated.

5. The chloride content of the blood corpuscles is nearly constant, about 0.31 per cent, the only serious divergence being found in pernicious anemia, where the average content is calculated to be 0.23 per cent.

6. An analysis of the blood-chloride percentage generally does not give more information than a simple cell volume determination. Only blood-chloride percentages which vary distinctly from the values calculated by formula (2) may be considered pathological.

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A NEW METHOD FOR THE DETERMINATION OF THE FIBRIN PERCENTAGE IN BLOOD AND PLASMA.

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The variations in the fibrin (fibrinogen) content of the blood have interested several observers, though the technique for these determinations has never been very good or convenient.

The spontaneously coagulable matter of the blood may be determined in two ways, by precipitating either the fibrinogen as such or as fibrin by the natural process of clotting. The last named method is the oldest and has generally been carried out so that the fibrin has been whipped out of a measured or weighed amount of blood (Hoppe-Seyler, 1; Samuely, 2), or the blood has been shaken with small rods and the fibrin adhering to these removed (Dastre, 3). A still older method used by Andral (4) and others was to leave a measured quantity of blood to clot, then whipping out and washing the fibrin ("*fibrine du caillot*"). This procedure is described by Doyon, Morel, and Kareff (5).

All these methods require a rather large quantity of blood (20 to 100 cc.), and they are not very accurate since the fibrin obtained is not pure and small amounts may be lost during the whipping and washing. A better way of determining the fibrin has been chosen by the authors, using a stable plasma for analysis because this may be freed of leucocytes. Often, however, this was not done. Pfeiffer (6) determined the fibrin by recalcifying oxalated plasma and calculating the fibrin percentage from the difference in the nitrogen content of plasma and serum. As it is necessary to know the cell volume for these calculations, it was either arbitrarily fixed as 40 per cent or was estimated by the method of Bleibtreu.

Cullen and Van Slyke (7) have proposed to isolate the clot from recalcified oxalate plasma and to determine its nitrogen content; the fibrin percentage is then calculated by multiplying by 6.25. It must also be mentioned that Bang (8) has proposed to determine the fibrin in blood by weighing 1 drop absorbed on filter paper and making a micro-analysis of its nitrogen content after extracting with a highly diluted alkaline solution.

The possibilities of fibrinolysis (Green, 9; Goodpasture, 10), which in my opinion are only to be feared when one does not use a cell-free plasma, have caused a great number of authors to prefer to determine the *fibrinogen*. For this purpose a stable plasma of some sort must be used.

Reyhe (11) used 12 cc. of fluoride plasma in which the fibrinogen was precipitated by adding 30 cc. of distilled water and 16 cc. of an ammomium sulfate solution having a specific gravity of 1.245. The precipitated fibrinogen was washed with an identical dilution of this salt and then with hot water, alcohol, and ether, and is weighed after drying. Doyon, Morel, and Péju (12) recommended the use of 1 cc. of 10 per cent acetic acid to 12 cc. of plasma for the same purpose.

The specific coagulation temperature of the fibrinogen has been used by Frédérick (13) and later by Whipple (14), who heated 20 cc. of clear, oxalated plasma to 56–60°C. for 10 minutes, and washed and weighed the precipitate.

With such methods the different authors have found very varying results (Table I), which may partly be explained by the physiological variations in the fibrin content; the number of observations in each series has often been very small.

A practically useful method for determining the fibrin (fibrinogen) percentage must fulfill the following conditions: (1) Quantities of blood must be used which may be easily obtained and several times without inconvenience to the patients. (2) A determination of the percentage both in plasma and in blood must be allowed. (3) The complete precipitation of the fibrin (fibrinogen) must be controlled. (4) Fibrinolysis must be excluded. (5) The fibrin and fibrinogen must be pure and cellular elements, which may either increase the weight or cause fibrinolysis (Rulot), should not be included.

TABLE I.

Fibrin and Fibrinogen Percentages in Blood and Plasma of Normal Men and Animals.

Author.	Technique.	Animal.	Fibrinogen or fibrin.	Percentage in plasma.	Percentage in blood.
Reyhe (11)	See text.	Cattle.	Fibrinogen.	0.41	
Müller (15)	Reyhe's.	Rabbits.	"	0.47	
Frédérick (13)	Heat coagulation.	Horses.	"	0.29–0.43	
Whipple (14)	" "	Men.	"	About 0.40.	
Whipple, Mason, and Peightal (16)	" "	"	"	0.30–0.40	
Pfeiffer (6)	See text.	"	Fibrin.	<0.31	
Bunge (17)	Whipping.	"	"		0.10–0.40
Richet (18)	"	"	"		0.19–0.28
Erben (19)	"	"	"		<0.30
Arthus (20)	"	"	"		0.10–0.20.
Arronet (21)	"	"	"		About 0.20.
Schneider (22)	"	"	"		" 0.20.
Biernacki (23)	"	"	"		" 0.19.
Becquerel and Rodier (24)	"	"	"		" 0.22.
Berggrün (25)	"	Children.	"		0.29–0.42
Krüger (26)	"	New-born.	"		0.12
Meek (27)	"	Dogs.	"		0.17–0.22
Andral (4)	Fibrine du caillot.	Men.	"		0.25–0.35

I shall now describe the technique for determining the fibrin content followed by me and afterwards the experiments, which have proved its reliability.

About 4.5 cc. of venous blood are run into a graduated 5 cc. centrifuge tube (Oluf Thomsen) divided into one-tenth of a cc. and containing 0.5 cc. of 3 per cent sodium citrate. The citrated blood is shaken and the blood adhering to the cork and upper part of the glass is wiped off.

After the specimen has stood for some time the corpuscles have sedimented sufficiently to draw off 0.025 cc. of plasma for the platelet count (Thomsen, 28; Gram, 29, 30) and 0.4 cc. for the determination of the coagulation time (Gram, 31). The velocity of this sedimentation depends upon the cell volume percentage and the fibrinogen percentage in the plasma (Gram, 32).

The specimen is then centrifuged for 90 minutes at 3,000 revolutions per minute and stopping very slowly. The tubes must be securely fixed in the corresponding holes of the centrifuge.

The amount of citrated blood and precipitate is noted, the cell volume being calculated by the equation

$$\text{Volume percentage} = \frac{P \cdot 100}{B}$$

P = Precipitate in cc.
B = Blood in cc.

The clear cell-free citrated plasma is drawn off with a pipette, and 2 cc. are transferred into a 50 mm. wide cylindrical vessel, whose bottom is slightly rounded off against the sides. 9 cc. of 0.9 per cent sodium chloride and 2 cc. of 1 per cent $CaCl_2 \cdot 6H_2O$ solution are then added and the vessel is placed in the thermostat at 35° C. for $1\frac{1}{2}$ hours. When the glass is taken out the contents will always be found clotted, except possibly in very severe cases of hemophilia or melena neonatorum.

By inclining and turning the glass around the clot will always loosen completely, not even leaving traces on the vessel.

It is thrown out on several layers of filter paper on which it forms a jelly-like cake. The water is absorbed very quickly by the paper, leaving a round shining membrane, which may easily be detached with a glass rod when the top layer of paper is thrown into a jar of water.

The detached membrane is kept in distilled water for 15 minutes and is then transferred into absolute alcohol for 5 minutes and finally into ether for another 5 minutes to dehydrate and extract lipoids.

The hardened, dehydrated fibrin which resembles a piece of paper is gripped by small wire pincers of known weight and hung in the thermostat for some hours or in an oven for a shorter period.

When the weight is constant the fibrin is weighed either on the analytical balance or on a fine torsion balance. The balance generally used only gave an accuracy of $\frac{1}{4}$ mg.

In the cylindrical glass vessel there is always left a small amount of liquid (diluted serum), which may be used as a control, since clotting either spontaneously or by addition of a little serum shows that the precipitation of the fibrinogen has not been complete. This control test is very fine, giving positive results with quantities of fibrin too small to influence the weight; *i. e.*, less than $\frac{1}{4}$ mg.

When the recalcified plasma was kept in the thermostat for the usual length of time the control never was found positive even in hemophilia.

In order to calculate the fibrin percentage in plasma (F_p) and blood (F_b) a knowledge of the following values is necessary:

Wf_2 = Weight in gm. of fibrin in 2 cc. of citrated plasma.
Cb = Citrated blood (0.5 cc.).
C = Citrate (0.5 cc.).
P = Cell precipitate in cc.

The formula for calculating the percentage in pure plasma is:

$$F_p = \frac{(Cb - P) \cdot Wf_2 \cdot 100}{(Cb - C - P) \cdot 2}$$

The corresponding formula for the percentage in pure blood is:

$$F_b = \frac{(Cb - P) \cdot Wf_2 \cdot 100}{(Cb - C) \cdot 2}$$

The technique in the first instance has been based upon a study of the coagulation time of citrated plasma on recalcification which has been published elsewhere (Gram, 31).

The exactitude of the cell volume determinations has been dealt with in another publication (Norgaard and Gram, 33).

In this paper we shall, therefore, only present the experiments concerning: (1) the proper recalcification of the citrated plasma; (2) the possibilities of fibrinolysis and fibrinogenolysis; (3) the accuracy of the method and the mean error on it; and (4) com-

parisons between my technique and the results gained by simple defibrination of blood and by determination of the fibrinogen after Whipple's method of heat coagulation.

Proper Recalcification.—1 drop of 1 per cent $CaCl_2 \cdot 6H_2O$ solution to 0.1 cc. of plasma equals 1 cc. of the solution to 2 cc. of plasma, since the pipette used in the coagulation experiments gave 20 drops to the cc.

It was found that the optimal recalcification of citrated plasma from a mixture of 0.5 cc. of 3 per cent citrate + 4.5 cc. of blood was

TABLE II.

Effects of Various Recalcification of Plasma Kept in the Thermostat for 1½ Hours After Recalcification.

Specimens.	I		II		III		IV		V	
Recalcification per 2 cc. of citrated plasma.	Fibrin percentage in plasma.	Control.	Fibrin percentage in plasma.	Control.	Fibrin percentage in plasma.	Control.	Fibrin percentage in plasma.	Control.	Fibrin percentage in plasma.	Control.
cc.										
0.5			0.32	–	0.31	+	0.51	–	0.39	–
1.0	0.33	–	0.32	–	0.34	–	0.51	–	0.37	–
1.5	0.33	–	0.32	–	0.34	–	0.49	–	0.39	–
2.0	0.33	–	0.32	–	0.34	–	0.49	–	0.39	–
2.5	0.33	–	0.32	–	0.33	–	0.51	–	0.37	–
3.0	0.32	–	0.32	–	0.34	–	0.51	–	0.39	–
3.5	0.33	–	0.32	–	0.34	–	0.49	–	0.37	–
4.0	0.32	–	0.32	–	0.34	–	0.51	–	0.33	+
4.5	0.33	–	0.32	–						
5.0			0.32	–						

1 to 4 cc. of 1 per cent $CaCl_2 \cdot 6H_2O$ per 2 cc. The effects of a varying recalcification are found in Table II.

In two cases the coagulation is not complete after 1½ hours, the recalcification in these specimens being respectively 0.5 and 4 cc. per 2 cc. of citrated plasma. With recalcifications between 1 and 3.5 cc. of 1 per cent $CaCl_2 \cdot 6H_2O$ the result is always the same except for the experimental error.

A recalcification of 2 cc. of 1 per cent $CaCl_2 \cdot 6H_2O$ per 2 cc. of citrated plasma must be considered safe, which also has been proved in practice.

TABLE III.

Fibrin Percentages in Equally Recalcified Specimens from the Same Individual Left for Various Periods in the Thermostat.

Time after recalcification (2cc. of 1 per cent $CaCl_2 . 6H_2O$).	Fibrin percentage in plasma.	Control.
min.		
5	No coagulation.	++
10	" "	++
15	0.09	++
20	0.32	+
25	0.32	−
30	0.33	−
35	0.32	−
60	0.32	−

TABLE IV.

Double Specimens, of Which No. I Is Left for 1½ Hours, and No. II for 24 Hours in the Thermostat after Recalcification.

Specimen No.	Diagnosis.	Fibrin percentage in plasma. I	II	Difference.
1	Convalescence after pneumonia	0.39	0.39	0
2	Rheumatic fever	0.55	0.55	0
3	Carcinoma of the liver	0.72	0.72	0
4	Normal	0.32	0.32	0
5	Lymphatic leucemia	0.32	0.32	0
6	Polycythemia	0.32	0.32	0
7	"	0.33	0.33	0
8	"	0.25	0.25	0
9	Febricula	0.55	0.55	0
10	" Stenosis pylori	0.82	0.82	0
11	Chronic polyarthritis	0.65	0.65	0
12	Cirrhosis of the liver	0.18	0.18	0
13	Pernicious anemia	0.18	0.18	0
14	Normal	0.25	0.25	0
15	Fatty degeneration of the liver	0.08	0.08	0
16	Polycythemia	0.30	0.30	0
17	Chronic constipation	0.29	0.31	+0.02
18	Neurasthenia	0.28	0.27	−0.01
19	Chronic nephritis	0.49	0.47	−0.02
20	Splenectomy (postoperative)	0.43	0.44	+0.01
21	Stenosis pylori	0.37	0.39	+0.02
22	Tertiary syphilis	0.66	0.63	−0.03
23	Fatty degeneration of the liver	0.12	0.13	+0.01
24	Pseudoleucemia	0.39	0.41	+0.02
25	Carcinoma of the liver	0.07	0.07	0
26	Normal	0.27	0.26	−0.01

If we consider how coagulation progresses in a series of identical specimens of citrated plasma recalcified in the same way, we find the results shown in Table III.

The coagulation is complete after 25 minutes, and the control is fine enough to show a residue of fibrin, which is too small to influence the weight of the precipitated fibrin.

Possibilities of Fibrinolysis and Fibrinogenolysis by an Analysis of Double Specimens from the Same Person.—The first question

TABLE V.

Analysis of Double Specimens of Which No. I Is Centrifuged at Once and No. II after Standing 24 Hours at Room Temperature.

Specimen No.	Diagnosis.	Cell volume.		Fibrin percentage in plasma.		Difference.
		I	II	I	II	
		per cent	*per cent*			
1	Normal	37	39	0.27	0.27	0
2	"	41	41	0.23	0.23	0
3	Febricula (cancer?)	40	40	0.54	0.54	0
4	"	43	42	0.62	0.62	0
5	Convalescense after rheumatic fever	38	38	0.32	0.32	0
6	Pernicious anemia	29	29	0.22	0.22	0
7	Nephritis; anemia	23	23	0.66	0.66	0
8	Enteritis	40	41	0.28	0.28	0
9	Pernicious anemia	27	27	0.24	0.24	0
10	Enteritis	43	44	0.33	0.33	0
11	Normal	44	46	0.35	0.36	+0.01
12	Abdominal carcinoma	44	45	0.52	0.54	+0.02
13	Renal tumor	26	26	0.62	0.60	−0.02
14	Constipation	39	39	0.28	0.29	+0.01
15	Normal	42	42	0.28	0.30	+0.02

was settled by letting Specimen I stand for 1½ hours and No. II for 24 hours in the thermostat after recalcification (Table IV).

This shows that an appreciable fibrinolysis does not take place in cell-free plasma within 24 hours. Specimens 12, 13, 15, 23, and 25 show that the low fibrin percentage found in some diseases of the liver is not due to fibrinolysis.

In the same way a series of double specimens (Table V) showed that a dissolution of the fibrinogen did not take place, when a specimen of citrated blood was left at room temperature for 24 hours before being centrifuged.

Error of the Method.—First, a series of double specimens of citrated blood were analyzed and showed very concordant results (Table VI).

TABLE VI.

Analysis of 29 Simple Double Specimens.

Specimen No.	Diagnosis.	Cell volume. I	Cell volume. II	Fibrin percentage in plasma. I	Fibrin percentage in plasma. II	Difference.
		per cent	*per cent*			
1	Normal	40	40	0.22	0.22	0
2	Enterocolitis	39	41	0.32	0.32	0
3	Polycythemia	67	67	0.32	0.31	0.01
4	Diabetes	40	40	0.38	0.38	0
5	Ulcus ventriculi	42	43	0.32	0.32	0
6	Normal	43	43	0.32	0.32	0
7	Ischias	40	41	0.32	0.32	0
8	Bronchitis	44	44	0.46	0.44	0.02
9	Nephritis	46	45	0.56	0.59	0.03
10	Febricula	39	40	0.82	0.82	0
11	Nephritis	36	36	0.28	0.28	0
12	Pregnancy	32	31	0.49	0.47	0.02
13	Normal	46	48	0.31	0.32	0.01
14	Nephritis	46	46	0.58	0.56	0.02
15	"	49	50	0.59	0.61	0.02
16	Polycythemia	72	72	0.37	0.37	0
17	Chronic polyarthritis	41	41	0.38	0.37	0.01
18	Cirrhosis of the liver	37	37	0.25	0.25	0
19	Pleurisy	37	39	0.66	0.68	0.02
20	Normal	47	49	0.30	0.32	0.02
21	Influenzal pneumonia	42	43	0.48	0.49	0.01
22	Nephritis(?)	50	50	0.90	0.90	0
23	Rheumatic fever	34	34	0.94	0.93	0.01
24	Convalescence after rheumatic fever	41	40	0.35	0.35	0
25	Influenzal pneumonia; fatty degeneration of the liver	44	44	0.39	0.40	0.01
26	Pleurisy	42	42	0.50	0.49	0.01
27	Influenza	48	49	0.33	0.32	0.01
28	Rheumatic fever	42	40	0.56	0.57	0.01
29	Normal	48	47	0.35	0.345	0.005

Then in three cases the mean error was determined by analysis of respectively 9, 9, and 10 specimens (Table VII-A, B, and C).

TABLE VII-A.

Convalescence after Angina.

Specimen No.	Cell volume.	Fibrin percentage in plasma.	Fibrin percentage in blood.
	per cent		
1	50	0.3745	0.1865
2	49	0.3707	0.1883
3	49	0.3543	0.1806
4	49	0.3548	0.1798
5	49	0.3712	0.1892
6	50	0.3575	0.1780
7	50	0.3756	0.1875
8	49	0.3712	0.1892
9	49	0.3875	0.1949

The mean error is calculated by the formula:

$$E = \pm \sqrt{\frac{\Sigma \cdot d^2}{n-1}}$$

E=mean error
d=variations of each determination from the average
n=number of determinations

The mean error on the fibrin percentage in plasma was ± 0.011, in blood ± 0.0055.

TABLE VII-B.

Graves' Disease (Severe Case).

Specimen No.	Cell volume.	Fibrin percentage in plasma.	Fibrin percentage in blood.
	per cent		
1	44	0.4075	0.2276
2	44	0.4075	0.2276
3	44	0.3912	0.2185
4	44	0.4075	0.2276
5	44	0.4081	0.2294
6	44	0.4075	0.2276
7	44	0.4075	0.2276
8	45	0.4112	0.2256
9	44	0.3912	0.2185

According to the above equation the mean error on the fibrin percentage in plasma was ± 0.0075, in blood ± 0.0041.

TABLE VII-C.

Normal Individual.

Specimen No.	Cell volume.	Fibrin percentage in plasma.	Fibrin percentage in blood.
	per cent		
1	39	0.2840	0.1725
2	40	0.2683	0.1622
3	40	0.2667	0.1600
4	41	0.2691	0.1595
5	40	0.2675	0.1611
6	40	0.2667	0.1600
7	41	0.2815	0.1652
8	40	0.2807	0.1678
9	40	0.2675	0.1611
10	39	0.2807	0.1715

According to the above equation the mean error on the fibrin percentage in plasma was ± 0.0073, in blood ± 0.0048.

My Technique for the Fibrin Determination Has Been Compared with a Determination of the Fibrin Percentage by Whipping Out the Fibrin (Table VIII).—The following technique was used: By venous puncture a specimen of 4.5 cc. was taken into citrate and treated in the ordinary way. Then 50 cc. of blood were placed in a graduated glass. This quantity is whipped for 10 minutes with a glass rod whose end is slightly widened.

TABLE VIII.

Fibrin Determinations by My Technique (I) Compared with Fibrin Determinations by Simple Whipping (II).

Specimen No.	Diagnosis.	Cell volume. I	Fibrin percentage in plasma. I	Fibrin percentage in blood.		Difference between fibrin percentage in blood. (II-I)
				I	II	
		per cent				
1	Polycythemia....	71	0.25	0.07	0.09	+0.02
2	Nephritis........	47	0.59	0.31	0.34	+0.03
3	"	36	0.28	0.18	0.16	−0.02
4	"	49	0.59	0.31	0.34	+0.03
5	Polycythemia....	73	0.36	0.10	0.12	+0.02
6	Neurasthenia....	44	0.33	0.18	0.18	0
7	Observation	49	0.37	0.19	0.19	0
8	Normal..........	40	0.25	0.15	0.14	−0.01
9	"	48	0.30	0.16	0.16	0

One then adds 5 cc. of fresh serum and whips for another 5 minutes. The defibrinated blood after standing for an hour is diluted with water and examined for clots, which may have been detached during the whipping or have been formed later.

The fibrin has collected itself as a reddish tube around the rod and may easily be slid over the top of it. It is washed in physiological saline solution till it is nearly colorless and then successively in water, hot distilled water, hot alcohol, and ether.

After drying to constant weight, the weight of fibrin in grams multiplied by 2 gives the fibrin percentage in blood.

TABLE IX.

A Comparison between Fibrin Determinations by My Method (I) and Fibrinogen Determinations by the Method of Whipple (II).

No.	Diagnosis.	Cell volume. I	Fibrin percentage in plasma. I	Fibrinogen percentage in plasma. II	Variation between fibrin and fibrinogen percentage in plasma. (II-I)
		per cent			
1	Nephritis	41	0.53	0.56	+0.03
2	"	49	0.59	0.60	+0.01
3	Febricula	52	0.45	0.50	+0.05
4	Diabetes	41	0.33	0.35	+0.02
5	Febricula; acromegalia	36	0.39	0.42	+0.03
6	"	46	0.46	0.52	+0.06
7	Pernicious anemia	27	0.23	0.24	+0.01
8	Normal	44	0.25	0.29	+0.04
9	"	43	0.31	0.36	+0.05

Several specimens had to be rejected either because particles of the clot were detached or because the clot would not lose its color and so might be supposed to contain other elements than pure fibrin.

The determinations shown in Table VIII gave very concordant results. It must, however, be noticed that the values compared are the smaller fibrin percentages in blood, so that the differences would be larger if the percentages in plasma could be compared. A comparison between my technique for the fibrin determination and a determination of fibrinogen by the method of *Whipple* gave the results shown in Table IX.

Instead of oxalated plasma I used 20 cc. of citrated plasma, which were heated to 56–60° C. for 10 minutes. The precipitated fibrinogen was washed, dried, and weighed and the calculation of the percentage was made in the same way as by my own method.

From the lowest to the highest fibrin (fibrinogen) contents there was a very good concordance between the results of the two methods, though the fibrinogen percentages on the average were somewhat higher.

The results in pathological cases[1] have been published in brief elsewhere (Gram, 32), so I shall give only the results of a series of observations o normal men and women.

TABLE X.

25 Normal Men.

	Fibrin percentage in plasma.	Fibrin percentage in blood.	Cell volume.
			per cent
Highest	0.36	0.19	51
Lowest	0.20	0.11	43
Average	0.27	0.14	48

TABLE XI.

25 Normal Women.

	Fibrin percentage in plasma.	Fibrin percentage in blood.	Cell volume.
			per cent
Highest	0.38	0.21	45
Lowest	0.21	0.12	37
Average	0.29	0.17	41

The fibrin percentage in plasma is slightly lower in men than in women. The difference between the percentage per 100 cc. of blood is even larger, owing to the larger average cell volume in men's blood.

The examination of the fibrin content in cases of anemia and polycythemia has shown that the percentage in plasma is the value kept constant during variations in the cell volume, so that the percentage per 100 cc. of blood is abnormally high in anemia and abnormally low in polycythemia.

[1] Hyperinosis in infectious diseases, cancer, pregnancy, nephritis, chronic polyarthritis, etc. Hypinosis only in severe degeneration of the liver. The results will appear in full in the *Acta med. Scandinav.*

TABLE XII.

Repeated Analysis for Fibrin in 8 Normal Persons (Nos. 2 and 8 Are Men, the Rest Are Women).

No.	Date.					Fibrin percentage in plasma. Fibrin percentage in blood.					Greatest variation from average.	Cell volume.				
	I	II	III	IV	V	I	II	III	IV	V		I	II	III	IV	V
1	Jan. 22	Jan. 29	Feb. 5	Feb. 12	Interval of 1 yr.	0.38 0.21	0.38 0.22	0.35 0.19	0.345 0.20	0.37 0.21	0.02	44	43	45	43	42
2	" 24	" 31	" 6	" 12		0.35 0.18	0.33 0.17	0.35 0.18	0.33 0.17		0.01	48	47	49	48	
3	" 20	Feb. 1	" 8	" 12	" " 1 "	0.33 0.19	0.33 0.19	0.30 0.16	0.29 0.17	0.31 0.18	0.02	41	43	44	42	42
4	" 20	" 1	" 4	" 11	" " 1 "	0.32 0.20	0.34 0.21	0.33 0.20	0.30 0.18	0.31 0.19	0.02	37	38	40	39	38
5	" 20	Jan. 27	" 3	" 10	" " 1 "	0.27 0.165	0.24 0.15	0.27 0.16	*	0.29 0.18	0.03	38	39	40	38	37
6	" 20	" 27	" 3	" 10		0.23 0.14	0.26 0.15	0.27 0.15	0.24 0.14		0.02	40	42	43	42	
7	" 21	" 28	" 3	" 10		0.22 0.13	0.25 0.15	0.24 0.15	0.24 0.14		0.02	40	41	42	40	
8	" 24	" 31	" 7	" 12	" " 1 "	0.20 0.11	0.22 0.11	0.23 0.12	0.20 0.11	0.21 0.12	0.02	44	46	49	46	43

*At the fourth examination No. 5 suffered from a cold. The specimen nevertheless was analyzed and showed a slight, not absolute increase; fibrin per cent in plasma 0.35, in blood 0.22. This result, of course, cannot be used as normal, since all infections will produce a more or less pronounced hyperinosis.

It has also to be settled whether the large variations in the normal fibrin percentage occur in the same person or whether each normal person has a certain level of fibrin content, which is kept fairly constant.

The last seems to be the case according to the results given in Table XII.

The fibrin was determined on six women (Table XIII) at different times of the day in order to see whether any variations could be traced or whether the digestive leucocytosis did influence the fibrin content.

This does not seem to be the case, the variations being always very small and independent of the digestive leucocytosis, which is very pronounced in the cases of Nos. 3, 5, and 6.

TABLE XIII.

Fibrin Percentage in Plasma, Cell Volume Percentage, and Number of Leucocytes at Different Times of the Day.

No.	Fibrin percentage in plasma.			Cell volume.			Leucocytes per c.mm.		
	10 a. m.	2 p. m.	6 p. m.	10 a. m.	2 p. m.	6 p. m.	10 a. m.	2 p. m.	6 p. m.
				per cent	*per cent*	*per cent*			
1	0.33	0.33		39	38		7,500	7,100	
2	0.32	0.32		42	41		5,900	7,700	
3	0.33	0.33		38	40		6,500	11,700	
4	0.29	0.31		42	42		8,200	9,900	
5	0.35	0.36	0.37	44	43	43	6,500	10,900	11,800
6	0.21	0.22	0.21	42	40	41	5,400	5,700	9,100

SUMMARY.

1. A technique is described by which the fibrin percentage in blood and plasma may be determined by recalcification of 2 cc. of clear cell-free plasma obtained from 5 cc. of citrated blood (0.5 cc. of 3 per cent citrate + 4.5 cc. of blood). This includes a determination of the cell volume.

2. On the same specimen one may determine the platelet count by Thomsen's method and the coagulation time by a method indicated by the author.

3. The technique described is shown to fulfill the conditions exacted for a trustworthy method.

4. The results by this technique in the blood of normal men and women are put forward. When the stress is laid upon the fibrin percentage per 100 cc. of plasma, this is caused by the results found in diseases of the blood which together with other pathological results have been published in brief elsewhere.

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A FURTHER STUDY OF THE RESPIRATORY PROCESSES IN MYA ARENARIA AND OTHER MARINE MOLLUSCA.*

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INTRODUCTION.

In a preliminary paper (1) the conclusion was tentatively advanced that *Mya arenaria* is a facultative anaerobic organism. This conclusion was reached as a result of repeated demonstration of the fact that this pelecypod continues to produce carbon dioxide in considerable amounts and for long periods when kept under anaerobic conditions. Further investigation has shown that various other bivalved forms and also certain of the gastropods continue to excrete, or to store and excrete carbon dioxide when the supply of oxygen in the medium is at a minimum. The object of the present investigation was to study quantitatively this apparent manifestation of anaerobiosis in such highly organized forms. It was thought that quantitative data relating to rates of oxygen absorption under normal or experimental conditions, to the hydrogen ion concentration maintained in the celomic fluid under various circumstances, and to rates of carbon dioxide excretion under anaerobic conditions should throw some light on the problem. Berkeley (2) has recently confirmed Collip's observations on carbon dioxide production in *Mya arenaria* under anaerobic conditions, and has demonstrated the same phenomenon in the two species *Saxidomus gigantea* and *Paphia staminea*. He has endeavored to relate the process to the decomposition of glycogen with the production of methane and carbon dioxide. His results, however, showed that glycogen did not disappear at a

* This work was carried out under tenure of a Fellowship granted by The Rockefeller Foundation.

more rapid rate in the anaerobic specimens than in the aerated controls except in the case of *Saxidomus*. He concluded, therefore, that a disappearance of glycogen invariably accompanies anaerobiosis in *Saxidomus*, and that no disappearance of glycogen accompanies anaerobiosis in *Mya* and *Paphia*. It would seem most probable that the source of oxygen during exposure of a clam to anaerobic conditions is the same in one species as another. It would follow then from Berkeley's results that glycogen is not likely to be the source of all the oxygen which goes to form carbon dioxide when the medium is oxygen-free. In view of the possible significance attached to glycogen as relating to the process, determinations of this tissue constituent were made in certain instances.

Methods.

The methods employed were as follows:

Determination of the pH of Celomic Fluid.

The pH of the celomic fluid was determined colorimetrically. Phenol red was the indicator used. The celomic fluid was drawn off without loss of carbon dioxide and delivered under paraffin oil into distilled water containing the adequate amount of phenol red. A pipette of narrow bore and containing a little paraffin oil was thrust deep into the pericardium, after an opening had been made just lateral to the hinge. The celomic fluid was aspirated into the pipette and 1 cc. delivered into 15 cc. of distilled water. The method of Cullen was then followed.[1]

Oxygen Consumption.

The rate of oxygen absorption of clams was determined by the well known Winkler method.

Carbon Dioxide Production.

Carbon dioxide production was determined by the use of the Van Slyke (3) apparatus for determination of blood plasma carbon dioxide. The specimens to be studied were placed in fresh

[1] I am indebted to Dr. Cullen for furnishing me with the details of his method before publication.

sea water in museum jars of known capacity and then sealed under water. After a definite period of time had elapsed, the jar was opened, the sea water was drained off, and the clams were removed to an open dish, and allowed to drain further. The water which was thus collected was added to the larger fraction and the total volume determined. The clams were at once opened up and the interior of the shells freed of tissue. The whole contents of the shells were then drained, gentle pressure being used to expel the liquid portion. The fluid which was obtained in this manner was a mixture of true celomic fluid and of sea water trapped in the mantle cavities. The carbon dioxide content of the sea water used in the experiment was determined before and after, also the carbon dioxide content of the composite fluid expressed from the clam tissue was determined before on control specimens and after on the fluid obtained as described. The drained clam tissue was weighed. The volume of water displaced by the shells was determined and the volume displaced by the clams as a whole was obtained by subtracting from the volume capacity of the container used in the experiment the volume of water which was drained off from the clams when the experiment was concluded. Sufficient data were thus available to enable one to calculate the carbon dioxide production per 100 gm. of wet drained tissue per hour, a factor which was in every case determined and used for comparative purposes. The method just described is not without error, but it gives, nevertheless, approximate results. As was previously shown (1), the carbon dioxide produced by a clam when it is removed from its natural habitat may be in large part retained in the celomic fluid, becoming fixed as calcium bicarbonate. When one determines, therefore, the carbon dioxide content of the sea water or celomic fluid in an experiment, one is dealing with carbon dioxide fixed as bicarbonate for the most part. The carbon dioxide production is obtained by taking 50 per cent of that present in the noted bicarbonate increase. As the amount of free carbon dioxide is relatively very small in comparison with that held as bicarbonate no attempt was made to measure it directly. If the free carbon dioxide were determined also, the results throughout would be slightly higher.

The above method for determining carbon dioxide production was checked up by determining the carbon dioxide excretion

when clams were aerated with carbon dioxide-free nitrogen and the gas produced collected in standard baryta which was afterwards titrated to phenolphthalein with standard acid. The results checked satisfactorily.

Results.

The pH of the Celomic Fluid Under Anaerobic Conditions.

It was found that no appreciable change in the pH of the celomic fluid of *Mya* or *Venus* occurred when they were kept for days immersed in paraffin oil, in a nitrogen atmosphere, in sea water in a sealed container, or simply exposed to atmospheric air. The pH for both normal and experimental specimens as determined by the method outlined was 7.8 to 7.9. Under all the experimental conditions imposed, the total carbon dioxide of the celomic fluid increased several fold.

Rate of Oxygen Absorption.

Considerable variation was found in the rate of oxygen consumption at constant temperature. Some forms, such as *Venus mercenaria*, which are capable of closing the valves completely, seem at times to use little or no oxygen, but one can be assured that oxidation is going on in the specimen, due to the fact that one can demonstrate a constant production of carbon dioxide, which is under these circumstances retained in the celomic fluid. Mitchell (4) found that the oxygen absorption of *Venus* and the oyster was very low unless the valves were opened slightly and a current of water maintained throughout the mantle cavities. In the case of *Mya*, the rate of oxygen absorption, the temperature being kept constant, is largely determined by the position of the siphon and the rate of flow of water through it. It is quite possible for the form to draw its siphon in completely and when this is done the rate of oxygen absorption falls almost to zero. It is, however, only under exceptional circumstances that specimens of *Mya* do not ventilate to a fair degree, while quite the reverse is true of *Venus*. It was not uncommon to find the carbon dioxide content of the celomic fluid of specimens of *Venus mercenaria* kept in running sea water in the laboratory for a few days over 30 volumes per cent, whereas the carbon dioxide content of these

forms when they are perfectly fresh is 6.5 volumes per cent. *Mya arenaria*, on the contrary, may be kept in the laboratory in running sea water for days without the carbon dioxide increasing in amount in the celomic fluid. This is due solely to the fact that the former type closes its valves and thereby prevents a free exchange of gases through the membranes of the gills and mantle, while the latter adapts itself to the new conditions, and ventilates freely. It was found by Mitchell (4) that the rate of oxygen absorption by oysters and clams varied directly as the temperature. This has been confirmed for *Mya*. It is difficult, however, to relate the results obtained directly to temperature changes. For example, the oxygen absorption for six specimens of *Mya* at 14°C. was found to be 1.40 cc. per 100 gm. drained clam tissue per hour. The same clams were then placed in fresh sea water at 26°C., and the oxygen absorption rate was found to be 11.92 cc. per 100 gm. per hour. On being replaced in fresh sea water at 14°C., the rate of oxygen absorption fell to 7.93 cc. The estimation was for periods of 1 hour in each case. In this instance, the siphons were not extended at first, but were well extended at the higher temperature, and fairly well in the last determination. The highest rate of oxygen consumption observed for fresh specimens of *Mya* at 14°C. was 5.78 cc. per 100 gm. per hour, and the lowest 1.40 cc. per 100 gm. per hour. The usual result, however, for specimens with siphons moderately extended was between 3 and 4 cc. per 100 gm. per hour. The oxygen absorption by specimens of *Venus mercenaria* under laboratory conditions was in all instances almost negligible. This was due, as will be shown later, to the valves being kept tightly closed.

Effect of Cleansing the Shells upon the Rate of Oxygen Absorption.

Owing to the possibility of organisms attached to the shells using up oxygen, the rate of oxygen absorption for specimens of *Mya arenaria*, which had had the shells sterilized by sponging with an alcoholic solution of bichloride of mercury, was determined. For some hours following the cleansing of the shells the oxygen absorption was very low, being from 0.2 to 0.4 cc. per 100 gm. per hour. That the result was not due to the killing of organisms on the shell was shown by the fact that the rate of oxygen

absorption came back to normal in 2 days time. The specimens for a time immediately following the cleansing operation drew in their siphons as completely as possible, and as they did not ventilate the mantle cavity and the gills they did not absorb oxygen. The presence of traces of mercury in the water would no doubt be a factor in causing the siphons to be withdrawn for a long period. This would appear to be the case, because cleansing the shells by means of sand paper caused a comparatively slight fall in the oxygen consumption. In one experiment, the rate of oxygen consumption immediately following sand paper cleansing was 1.9 cc. per 100 gm. per hour, as compared with 3.17 cc. per 100 gm. per hour in the controls.

Oxygen Fixation by the Shell.

Mitchell (4) found that a certain amount of oxygen was fixed by the shells of clams and oysters. The amount of oxygen which was removed from fresh sea water by the uncleaned shells of specimens of *Mya* studied was found to be very small. Shells displacing 100 cc. of water would cause the oxygen of fresh sea water to disappear at the rate of 0.5 to 0.9 cc. per hour. For very detailed work on oxygen consumption, the effect of the shell must be determined and the results corrected accordingly, as Mitchell has pointed out.

Effect of Breaking the Shell upon the Rate of Oxygen Absorption.

If the valves of specimens of *Venus mercenaria* be carefully cracked along the margin, the rate of oxygen absorption rises immediately. The rate of oxygen absorption in one instance for specimens of *Venus* was 0.051 cc. per 100 gm. per hour, at 20°C., while the rate for specimens with shells cracked was 1.78 cc. per 100 gm. per hour. Similarly, specimens of *Mya* with a low rate of oxygen absorption, as a result of bichloride cleansing, can be made to absorb oxygen much faster by cracking the shells. In one such experiment, the rate of oxygen absorption in the bichloride cleansed specimen was 0.198 cc. per 100 gm. per hour. After cracking the shells and allowing ventilation of the mantle cavity, the rate rose to 0.822 cc. per 100 gm. per hour.

Rate of Carbon Dioxide Production under Anaerobic Conditions.

A large number of experiments were carried out to determine the rate of carbon dioxide production by specimens of *Mya arenaria* which were deprived of dissolved oxygen in the medium. These experiments were carried out under widely different conditions. At first, attempts were made to use oxygen-free sea water, but it was early noted that the sea water in which specimens were sealed up became oxygen-free within a very short period, especially if the volume of water was small. As many experiments were carried on for days and as the oxygen available in the medium would be removed within the first few hours, the error introduced by the use of this method was slight. The results of this series of experiments are shown in Table I. While there is a certain amount of variation in the rate of carbon dioxide production as determined by this method, yet this is not surprising, since the periods over which the anaerobic condition was maintained varied from a few hours to several days. Also the temperature could not

TABLE I.

CO_2 Production by Mya arenaria.

Temperature.......31°C.		20°C.		14°C.		8°C.	
Duration of experiment.	CO_2 per 100 gm. per hr.	Duration of experiment.	CO_2 per 100 gm. per hr.	Duration of experiment.	CO_2 per 100 gm. per hr.	Duration of experiment.	CO_2 per 100 gm. per hr.
hrs.	*cc.*	*hrs.*	*cc.*	*hrs.*	*cc.*	*hrs.*	*cc.*
4.50	3.60	21.50	2.44	46	1.95	18	1.26
6.50	3.86	18	2.97	70	2.52	21	1.12
5.00	5.59	17	2.30	122	1.53	191	0.87
4.40	4.37	19	2.34	124	1.60	192	0.84
		43	2.58	146	1.19	192	0.86
		13	2.53	44	1.41	192	0.99
		13	2.44	45	1.18		
		18	3.54	44.30	0.70		
		15	2.47	45	0.89		
		12	2.08	45	1.09		
		43	1.68	69	1.42		
		21	2.31	25	0.57		
		20	1.92	24	0.74		
		7	2.43				
Average.....	4.35		2.43		1.29		0.99

be maintained absolutely constant, there being a variation of at least 1 ±°C. The volume of water was, in some instances, large relative to the volume of clam tissue, while in other experiments the specimen jar was packed full of clams, and the water simply filled the interstices.

Effect of Temperature upon the Rate of Carbon Dioxide Production under Anaerobic Conditions.

The rate of carbon dioxide production under anaerobic conditions was determined at the following approximate temperatures: 8°C., the temperature of the ice box; 14°C., the temperature of running sea water in the laboratory at Saint Andrews; 20°C., the temperature of running sea water in the laboratory at Woods Hole; 31°C., the temperature of an improvised water thermostat. The metabolic rate, as is shown by the results recorded in Table I, is a direct function of the temperature.

Effect of Temperature upon Survival Time under Anaerobic Conditions.

A general correlation exists between the temperature and the length of time clams may be kept in good condition in an anaerobic medium. At 31°C., the survival time is about 24 hours; at 14°C., about 8 days; and at very low temperatures, the specimens may be kept for weeks.

Effect of Potassium Cyanide upon Oxygen Absorption and Carbon Dioxide Production.

It was found that the rate of oxygen absorption was depressed by the presence of a small amount of potassium cyanide in the medium. The rate of carbon dioxide production under anaerobic conditions was likewise depressed by potassium cyanide. A concentration of 1 part of KCN in 2,500 of sea water caused the CO_2 production to fall from 2.08 cc. per 100 gm. per hour, to 1.02 cc. Greater concentration depressed the CO_2 production still more, while lower concentrations had less effect.

A point of interest in the effect of potassium cyanide upon the clam is that it tends to lower the difference in the concentration of CO_2 in the celomic fluid and that of the outside water. Unless

an experiment were run for several days, it was found that the concentration of CO_2 in the celomic fluid was approximately 10 volumes per cent higher than that in the water of the container. In the presence of potassium cyanide, the rate of CO_2 production falls, and there is a tendency for the concentration of CO_2 in the medium to approach that maintained in the celomic fluid.

Effect of Submersion in Distilled Water upon the Rate of Carbon Dioxide Production under Anaerobic Conditions.

As was previously shown (1) clams may be kept in distilled water for varying periods and under these circumstances, they yield their salt to the water very slowly. The rate of carbon dioxide production by specimens of *Mya* kept in distilled water, was determined and it was found that the rate was greatly depressed. In one instance, a result of 1.02 cc. of CO_2 per 100 gm. per hour was obtained as compared with 2.44 cc. per 100 gm. per hour in the control. In *Sycotypus* the large whelk, the rate of CO_2 production fell in distilled water to 0.39 cc. per 100 gm. per hour from a normal of 1.10 cc.

Rate of Oxygen Absorption Following Exposure to Anaerobic Conditions.

The rate of oxygen absorption by specimens of *Mya* which had been kept for some days under anaerobic conditions was determined. It was found that immediately the specimens were transferred from the oxygen-free water to fresh sea water that the rate of oxygen absorption far exceeded the normal. Also the rate of absorption while far above normal at first, gradually fell until it reached the normal level. It required 3 days in some instances before the normal rate was regained. The results illustrating this effect are shown in Table II. The rate of oxygen absorption following an anaerobic period varied directly with the temperature. Thus in one instance, after 4 days of oxygen want, the rate of oxygen absorption at 5°C. was 7.06 cc. per 100 gm. per hour; at 14°C. it was 15.10 cc. per 100 gm. per hour; while at 22°C. it was 21.20 cc. per 100 gm. per hour.

TABLE II.

Oxygen Absorption Following Period of Anaerobic Condition.

Experiment.	Duration of anaerobic period.	Rate of oxygen absorption per 100 gm. per hr.	Remarks.
1	6 days at 14°C.	14.62	
2	1 " " 14 "	7.24	
3	2 " " 14 "	12.25	
4	4 " " 14 "	19.80	
5	4 " " 14 "	21.24	
6	4 " " 14 "	6.99	At 5°C.
		13.75	" 14°C.
7	6 days at 14°C.	17.60	
		8.46	Aerated 24 hrs.
8	3 days at 14°C.	19.61	
		11.50	Aerated 1 hr.
		11.26	" 2 hrs.
9	4 days at 14°C.	14.24	
10	3 " " 14 "	7.13	Aerated 3 hrs.
		3.20	" 20 "
		2.33	" 44 "
11	9 days at 8°C.	14.40	
		12.84	Aerated 1 hr.
		7.20	" 18 hrs.
		7.38	" 24 "
		3.24	" 48 "

Glycogen Content Before and After Periods of Oxygen Deficiency.

It was found that the glycogen content of specimens of *Mya arenaria* gradually fell after they were placed in the laboratory tanks. The glycogen content of perfectly fresh specimens calculated as per cent of wet drained tissue may run over 11 per cent. In specimens left in the laboratory tanks for 2 weeks, this was not observed to fall below 2 per cent. Specimens with a glycogen content of 2 per cent sealed up for a period of 6 days at a temperature of 18°C. were found to have only 0.25 to 0.30 per cent glycogen. Berkeley (2) has shown that the glycogen content of clams falls under anaerobic as well as aerobic conditions in the laboratory.

DISCUSSION.

The fact that no appreciable change in the hydrogen ion concentration of the celomic fluid could be detected even after several

days exposure of specimens of *Mya arenaria* and *Venus mercenaria* to abnormal conditions shows that these forms are capable of regulating the reaction of the body fluids quite as well as the higher forms. As was previously shown (1) the great increase in the carbon dioxide content of molluscan celomic fluid which is so readily brought about is due for the most part to increase in calcium bicarbonate. The source of the calcium is probably twofold; namely, the reserves in the liver and the shell itself. As a result of such forms being able to adjust their acid-base balance within very wide limits, a very simple method for determining metabolic rates under varying conditions is available.

It was thought probable that the cause of death in many marine forms on exposure to anaerobic conditions might be due not so much to oxygen deficiency, as to a change in body fluid reaction due to accumulation of acid end-products. Attempts were therefore made to prolong the survival time of many forms by adding precipitated calcium carbonate to the sea water in which the specimens were sealed up. Little success was attained however. The only forms which could be kept sealed up in oxygen-free sea water for long periods were the various calcareous shelled mollusks of both the pelecypod and gastropod types.

As calcareous shelled mollusks such as *Mya* continue to produce carbon dioxide at a fairly uniform rate under anaerobic conditions, and as this rate is directly dependent upon temperature and is depressed by the presence of potassium cyanide it is evident that oxidation still proceeds in the tissues even though the supply of oxygen from without is completely cut off. The question therefore arises: What is the source of this oxygen? Does it come from the breaking down of glycogen or some other food principle with the production of CO_2 and a paraffin compound such as methane, or is there some compound in the tissue of the nature of an organic peroxide which is capable of supplying oxygen as it is required, and which is itself reformed by the taking up of dissolved oxygen from the medium. The results which are herein reported, taken in conjunction with the negative findings of Berkeley as relating to a glycogen source for the oxygen would lend a measure of support to the latter hypothesis. It is difficult to determine with great accuracy the carbon dioxide production by specimens of *Mya* for very short periods of time, whereas oxygen absorption

can be measured with a fair degree of accuracy over periods of only a few minutes duration. There was no indication, however, in the experiments in which carbon dioxide production immediately following a period of oxygen want was determined, of a great increase in the rate of CO_2 production parallel with the greatly increased rate of oxygen absorption. This point was also tested in another manner. Three specimens of *Mya* were aerated with pure nitrogen gas for a period of 30 hours, the carbon dioxide which was excreted, being collected in standard baryta. Air rendered CO_2-free, was then passed through the system. The CO_2 production for the anaerobic periods was 2.60 cc. per 100 gm. per hour, while it increased to 4.27 cc. when the oxygen was restored. Unfortunately, due to lack of nitrogen gas, this method could not at this time be pursued further. As the normal rate of oxygen consumption was higher than the average rate of CO_2 production during periods of oxygen want it is probable that the metabolic rate may be slightly less in the latter case than in the former, and that a slight increase in the metabolic rate might therefore be expected, following the renewal of the oxygen supply. However, the rate of oxygen absorption for some time following renewal of the oxygen supply after anaerobic periods was far in excess of any observed increase in CO_2 production. Further data on the CO_2 production must be obtained to make this point absolutely definite. This phenomenon points, in our opinion, to the rebuilding of a peroxide in the tissue or to the recuperation of some substance which is the source of the oxygen during anaerobic periods.

It would appear then, while from a superficial view-point *Mya arenaria* and allied molluscan forms are facultative anaerobic organisms, that they are not anaerobic in the strict sense of the term. They can live for varying periods of time in the absence of outside oxygen, but their survival time while long, is nevertheless limited, and is directly proportional to the temperature. A possible explanation of the phenomenon of apparent anaerobiosis is that the tissues of these forms are capable of storing a large amount of oxygen which is readily available for the metabolic needs. The time of death under anaerobic conditions one would expect to be coincident with the disappearance of the oxygen store.

No evidence was obtained which would indicate the exact nature of the substance which takes up oxygen and holds it for the use of the tissues. That it is not in the celomic fluid or excreted to the water in the container was shown by delivering celomic fluid and water used in the experiment into water of known oxygen content and noting if any oxygen was fixed by the fluid added. The Winkler method was used and no appreciable change in oxygen content was demonstrated. It is therefore probable that the oxygen holding compound is present in the tissue only.

While the results so far obtained lead us to suggest the above explanation of the phenomenon, sufficient quantitative data have not yet been brought forward to justify one in saying that this is the only way in which the oxygen used by the clam tissue during periods of imposed anaerobic conditions is supplied.

CONCLUSIONS.

Calcareous shelled mollusks by virtue of being able to use the calcium reserves of the liver and the shell exhibit the power to regulate their acid-base equilibrium with such precision that no change in the pH index is brought about even when they are subjected to most abnormal conditions.

Mya arenaria, when placed under anaerobic conditions will survive for a period of time which is dependent upon the temperature. During this anaerobic period, carbon dioxide is produced at a uniform rate and this rate is accelerated by raising the temperature and depressed by lowering the temperature. The graph illustrating the temperature effect approximates to a straight line.

Glycogen disappears from the tissues during anaerobic periods. Potassium cyanide depresses the rate of carbon dioxide production under anaerobic conditions as well as depressing the oxygen consumption under normal circumstances. This points to the carbon dioxide production under anaerobic conditions as being due to a series of oxidations.

The rate of oxygen absorption immediately following imposed anaerobic periods is much higher than normal. The normal rate is regained gradually.

It is suggested that these forms have a store of available oxygen in their tissue which suffices to supply the necessary oxygen when the outside supply is cut off.

These forms are therefore unique in two respects: they can retain if necessary a large part of the carbon dioxide produced under aerobic or anaerobic conditions by virtue of their ability to adjust their acid-base equilibrium at widely varying levels; and they have a source of oxygen which is available in the tissue for metabolic needs during long periods of oxygen insufficiency in the enveloping medium.

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3. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
4. Mitchell, C., *Rep. U. S. Bureau Fisheries*, 1912, 207.

SULFATES IN BLOOD.

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Sulfates exist in blood in extremely minute amounts, and as the sulfate ion is not known to possess any striking physiological properties but little experimental work has been carried out on this phase of blood chemistry. Data concerning the sulfate content of blood, obtained by an analysis of the ash, include the sulfur present in combination in the protein molecule and have therefore but a limited usefulness in a study of the inorganic sulfur of this fluid. In fact, the only figures for inorganic sulfates of blood which I have been able to find after a search of the literature are those published by De Boer.[1] This investigator furnishes figures for horse blood, the SO_4 content of which he finds to be 0.02123 per cent, "an amount which is likely to vary in the case of different animals and more particularly in different periods of feeding." De Boer's results were obtained by means of a sedimentation method proposed by Hamburger[2] in which the volume of barium sulfate resulting from the treatment of the sulfate-containing liquid with hydrochloric acid and barium chloride is measured after centrifuging in a special calibrated tube. Protein-free filtrates were obtained by filtration through a celloidin filter under a pressure of five atmospheres. The determination of small amounts of sulfate by means of the turbidimeter has been in use among technical chemists for many years, and I have found it possible after a considerable amount of experimental work to apply the same analytical principle in the determination of sulfates in blood and serum, although on account of the exceedingly small amount of material to be deter-

[1] De Boer, S., *J. Physiol.*, 1917, li, 211.

[2] Hamburger, H. J., *Biochem. Z.*, 1916, lxxvii, 168.

mined it has been necessary to substitute the nephelometer for the turbidimeter.

Briefly stated the procedure consists in the removal of protein by means of a solution of mercuric chloride and hydrochloric acid, the formation of colloidal barium sulfate in the filtrate, and the measurement of the density of this precipitate by means of the nephelometer.

My preliminary experiments were carried out on beef blood, but when after a satisfactory technique had been attained I attempted to apply this method to human blood I was unable to obtain results, because the sulfate content of human blood is but a fraction of that contained in the blood of any species of animal examined.

The method as applied successfully to the blood of a variety of animals (see Table II) is as follows:

The blood is collected by venous puncture and coagulation prevented by means of powdered sodium citrate (30 mg. per 10 cc. of blood) in the manner usually followed in the case of specimens intended for chemical examination. To 10 cc. of blood or plasma contained in a 200 cc. Erlenmeyer flask are added an equal volume of 0.02 N hydrochloric acid and after an interval of 5 minutes 30 cc. of a 5 per cent solution of mercuric chloride containing 5 cc. of concentrated hydrochloric acid (sp. gr. 1.178) per liter. After vigorous shaking the mixture is allowed to stand for 1 hour and is then filtered through a dry 11 cc. filter paper. Filtration is fairly rapid and the filtrate should be absolutely clear. For this filtration the use of a high grade of "ashless" filter paper is essential; I have used Whatman's No. 44, which has been found free from all traces of sulfate.

For the determination of total inorganic sulfates 10 cc. of the clear filtrate (equivalent to 2 cc. of whole blood or plasma) are pipetted into a 100 cc. beaker, and to this are added 5 cc. of a 1.0 per cent solution of ammonium nitrate, and with stirring 5 cc. of a 1.0 per cent solution of barium chloride containing 5 cc. of concentrated hydrochloric acid per liter. After a period of 10 minutes the colloidal suspension of barium chloride is compared to a standard which has been prepared simultaneously with the unknown in the following manner: To 10 cc. of a standard solution of potassium sulfate (equivalent to 0.10 mg. of sulfur) are

added 10 cc. of the acid mercuric chloride solution, 10 cc. of 1.0 per cent ammonium nitrate, and 10 cc. of 5 per cent barium chloride.

To calculate the results divide the reading of the standard (usually 20) by the reading of the unknown and multiply the dividend by 50. This will give the result expressed as milligrams of sulfur per 100 cc. of blood.

To determine inorganic sulfates in normal human blood the procedure is as follows: 5 cc. of oxalated blood or plasma are treated with 5 cc. of 0.1 N hydrochloric acid, 5 cc. of 5 per cent mercuric chloride solution, and 0.3 gm. of finely powdered mercuric chloride. The mixture is then shaken vigorously for 5 minutes, and at intervals for 1 hour, and is then poured on a small dry "ashless" filter. 5 cc. of this filtrate, which should of course be absolutely clear, are treated with 1 cc. of a 1.0 per cent solution of ammonium nitrate and 1 cc. of the acidified barium chloride solution described above, and the turbidity so produced is compared after an interval of 10 minutes with a standard prepared by adding to 10 cc. of a standard solution of potassium sulfate 10 cc. of 5 per cent mercuric chloride solution, 4 cc. of 1.0 per cent ammonium nitrate, and 4 cc. of 1.0 per cent barium chloride solution.

While the technique described above gives excellent results with normal material it will be found that with pathological specimens the amount of sulfate present is sometimes so much increased that it is impossible to obtain a colloidal suspension of barium sulfate which will not precipitate. In such cases it is desirable if a sufficient amount of material is available to use the technique described for use with animal blood.

As the sulfate concentration of human blood shows greater variations than does the blood of animals it is usually desirable to make use of three standard solutions of potassium sulfate of which 10 cc. are equivalent to 0.1, 0.05, and 0.03 mg. of sulfur respectively.

Ammonium nitrate has been used because it facilitates the formation of colloidal suspensions of barium sulfate. Mercuric chloride was chosen after a trial of the more commonly used protein precipitants as it appears to be the only one of these reagents which does not in some way interfere with subsequent precipitations of barium sulfate.

All chemicals used should of course be tested for the presence of sulfates. All specimens of hydrochloric acid and ammonium nitrate examined have proved free from this impurity, but several samples of c. p. mercuric chloride have been found to contain appreciable amounts. Mercuric chloride can easily be purified so that it no longer gives precipitates with acidified barium chloride solutions by several (from three to five) recrystallizations from hot water.

The standard solutions of potassium sulfate were made from the recrystallized salt. It is most convenient to prepare a stock solution containing 5.4370 gm. of K_2SO_4 per liter, 1 cc. of which is equivalent to 1 mg. of sulfur, and from this strong solution to prepare by suitable dilution the three weaker standards.

The procedure described above is believed to furnish a method of measuring the inorganic sulfates of the blood, with an accuracy of approximately 95 per cent. It would seem probable that other forms of sulfate analogous to the ethereal sulfate and neutral sulfur of urine exist in blood.[3] I have carried out a number of experiments to obtain evidence of the presence of these forms of sulfur in blood but have invariably obtained negative results.

In an attempt to demonstrate the existence of ethereal sulfates in blood 10 cc. portions of the blood filtrate deproteinized by means of mercuric chloride were heated (after the addition of 1 cc. of concentrated hydrochloric acid) in a boiling water bath for periods of from $\frac{1}{2}$ to 2 hours. After cooling, an amount of alkali just sufficient to neutralize the acid was added and the mixture then treated with an acid solution of barium chloride. This procedure has been carried out on a considerable number of samples of both human and animal blood, but in no case have I been able to obtain evidence of the existence of conjugated compounds of sulfuric acid, as shown by an increase in the sulfates after the above acid treatment. In my search for the presence of sulfur compounds existing in the form of "neutral non-protein sulfur" I have oxidized portions of the mercuric chloride filtrate with potassium chlorate and with nitric acid, without, however, obtaining evidence of any increase in the amount of barium sulfate precipitate obtained after this oxidation.

[3] Kahn, M., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 139.

While the above results can scarcely be taken to furnish absolute and final proof of the non-existence of conjugated forms of

TABLE I.

Recovery of Potassium Sulfate Added to Blood.

Kind of blood.	S per 100 cc.			
	Inorganic sulfate in blood.	Sulfate added.	Theory.	Found.
	mg.	*mg.*	*mg.*	*mg.*
Beef	1.88	0.5	2.38	2.35
"	1.88	1.0	2.88	2.97
"	1.88	2.0	3.00	3.92
"	1.88	2.0	3.88	3.90
"	1.88	4.0	5.88	5.81
Human	0.9	0.3	1.20	1.18
"	0.9	0.5	1.40	1.38
"	0.9	1.0	1.90	1.90
"	0.9	4.0	4.90	5.00

TABLE II.

Inorganic Sulfates in the Blood of Animals.

Animal.	S per 100 cc. of blood.	Remarks.
	mg.	
Beef	2.6	Average of 18 specimens.
"	1.8	Minimum " 18 "
"	3.5	Maximum " 18 "
Horse 1	1.8	
" 2	3.1	
Sheep 1	2.5	
" 2	4.0	
" 3	2.8	
Hog 1	2.7	
" 2	2.5	
Rabbit 1	3.2	
Guinea pig 1	2.0	
Dog 1	2.5	
" 2	2.1	
" 3	3.5	

sulfur in blood, they at least indicate that if sulfur compounds of this type do exist in this fluid the amounts present must be exceedingly minute.

TABLE III.

Inorganic Sulfates in Human Blood.

No.	Diagnosis.	Inorganic SO_4. S per 100 cc. of blood.	Non-protein nitrogen per 100 cc. of blood.
		mg.	*mg.*
1	Normal.	0.5	
2	"	0.5	
3	"	0.6	
4	"	0.7	
5	"	0.7	
6	"	0.9	
7	"	1.0	
8	"	1.0	
9	"	1.0	
10	"	1.1	
11	Pregnancy.	1.3	30
12	"	1.0	28
13	Nephritis.	1.2	41
14	Pellagra.	0.7	25
15	Hyperthyroidism.	0.6	32
16	Cardiorenal disease.	0.7	32
17	Nephritis.	0.6	35
18	"	1.6	35
19	Leucemia.	7.0	68
20	Nephritis.	3.5	55
21	Epilepsy.	0.5	39
22	Intestinal obstruction.	3.0	45
23	Diabetes.	1.0	
24	Nephritis.	3.7	75
25	"	2.0	38
26	"	3.0	55
27	"	2.7	60
28	"	3.2	65
29	"	3.8	62
30	Cardiorenal disease.	1.2	40
31	" "	0.7	33
32	" "	0.8	33
33	" "	0.7	28
34	" "	1.0	35
35	Uremia.	5.0	174
36	"	4.6	80
37	"	6.2	75
38	"	8.0	142
39	"	12.5	187
40	"	12.0	210
41	"	16.0	200

In Table I are shown the results obtained when various amounts of potassium sulfate are added to beef and to human blood. Table II contains figures obtained on the blood of various animals and in Table III are collected the results of the examination of human blood, both normal and pathological.

SUMMARY.

The inorganic sulfates of blood, as determined by a new and simple method, a description of which is given is found to amount to from 1.8 to 4.0 mg. of sulfur per 100 cc. of blood in the case of various species of animals and from 1.0 to 0.5 mg. in normal human blood. In nephritics with nitrogen retention there is also found a retention of inorganic sulfate, figures as high as 12 and 16 mg. having been obtained.

Experiments made with a view to obtaining evidence of the existence in blood of conjugated compounds of sulfuric acid, and of bodies analogous to the neutral sulfur fraction of urine, have given negative results, indicating the probable non-existence of this class of bodies or the fact that they are present in amounts so minute that their presence cannot be detected by the methods now in use.

SOME OBSERVATIONS ON CREATINE FORMATION IN A CASE OF PROGRESSIVE PSEUDOHYPERTROPHIC MUSCULAR DYSTROPHY.

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In the course of a study of a series of myopathies, we found that creatine administered by mouth was in large part or completely eliminated in advanced, progressive pseudohypertrophic muscular dystrophy cases. This confirms the earlier observations of Levene and Kristeller[1] who estimated the creatine content of beef given such patients.

Recovery of considerable amounts of ingested creatine in established creatinurias has been reported by Krause[2] for normal children, by Powis and Raper[3] in myotonia congenita, and by Gamble and Goldschmidt[4] for normal infants. Our muscular dystrophy patients have shown a much diminished creatinine coefficient and a high degree of creatinuria; apparently the power to convert creatine, both preformed and produced in intermediate metabolism, is markedly impaired. We shall present elsewhere evidence that the abnormalities of carbohydrate metabolism in muscular dystrophy (Janney, Goodhart, and Isaacson;[5] McCrudden[6]) are not responsible for the creatinuria in the sense of the carbohydrate deficiency of Mendel and Rose.[7] We are convinced that such cases afford a unique opportunity to study the problems of creatine formation in man.

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[1] Levene, P. A., and Kristeller, L., *Am. J. Physiol.*, 1909, xxiv, 45.

[2] Krause, R. A., *Quart. J. Exp. Physiol.*, 1914, vii, 87.

[3] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 363.

[4] Gamble, J. L., and Goldschmidt, S., *J. Biol. Chem.*, 1919, xl, 199, 215.

[5] Janney, N. W., Goodhart, S. P., and Isaacson, V. I., *Arch. Int. Med.*, 1918, xxi, 188.

[6] McCrudden, F. H., *Arch. Int. Med.*, 1918, xxi, 256; *J. Am. Med. Assn.*, 1918, lxx, 1216.

[7] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213.

The controversy as to whether creatine formation is primarily exogenous or endogenous has yielded the following facts:

Creatinuria has been more easily established in normal adults (older children, women, adult males after creatine administration) on a high protein diet than on a low protein intake (Folin and Denis[8]; Denis and Minot[9]); however, negative results are reported (Rose[10]; Rose, Dimmitt, and Bartlett[11]; Stearns and Lewis[12]).

In established creatinuria, the creatine excretion is increased in going from a low protein to a high protein diet (Levene and Kristeller[1]; Talbot and Gamble[13]; Denis[14]; Denis and Kramer[15]; McCollum and Steenbock[16]; Steenbock and Gross[17]; Harding and Young[18]).

In established creatinuria, ingested creatine is largely or completely recovered in the urine, in part as creatinine (Levene and Kristeller; Krause; Powis and Raper; Gamble and Goldschmidt). Preformed creatine may account for part of the creatine elimination in supposed creatine-free diets, and must be taken into consideration (Gamble and Goldschmidt).

It is indicated, therefore, that creatine may have an exogenous as well as an endogenous origin. That is, it is a product of the catabolism of tissue, reserve, and food proteins. In the normal adult, creatine undergoes further change. It is not completely destroyed under conditions of carbohydrate deficiency (Mendel and Rose) or muscular insufficiency (Shaffer[19]), but is excreted into the urine as such and in smaller part as creatinine.

Attempts to find a precursor of creatine among the protein cleavage products have centered on the amino-acid arginine and related bases. Negative results are reported by von Hoogenhuyze and Verploegh[20] following the ingestion of casein and gelatin, proteins relatively rich in arginine. Jaffé[21] and also Baumann[22,23] injected arginine into animals without finding an in-

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[8] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.

[9] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561.

[10] Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.

[11] Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918, xxxiv, 601.

[12] Stearns, G., and Lewis, H. B., *Am. J. Physiol.*, 1921, lvi, 60.

[13] Talbot, F. B., and Gamble, J. L., *Am. J. Dis. Child.*, 1916, xii, 333.

[14] Denis, W., *J. Biol. Chem.*, 1917, xxix, 447; xxx, 47.

[15] Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.

[16] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.

[17] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265.

[18] Harding, V. J., and Young, E. G., *J. Biol. Chem.*, 1920, xli, p. xxxv.

[19] Shaffer, P. A., *Am. J. Physiol.*, 1908–09, xxiii, 1.

[20] von Hoogenhuyze, C. J. C., and Verploegh, H., *Z. physiol. Chem.*, 1908, lvii, 161.

[21] Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.

[22] Baumann, L., and Marker, J., *J. Biol. Chem.*, 1915, xxii, 49.

[23] Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1918, xxxv, 75.

crease in creatine formation. Thompson[24] has reported numerous experiments in the course of which arginine was injected into rabbits, dogs, and ducks; he was able to observe an increase in creatine excretion and the muscle content in creatine in most instances. Myers and Fine[25] obtained a higher muscle content in creatine for rats fed on the arginine-rich protein edestin. Inouye[26] observed some formation of creatine from arginine added to liver extracts, and in liver perfusion experiments. Jaffé showed that glycocyamine might be converted into creatinine and creatine in experiments with rabbits; Doerner[27] confirmed this, finding also that glycocyamidine was changed to creatinine. Mellanby[28] obtained no effect when glycocyamine was fed to rabbits and fowls. Baumann and Hines[29] found no evidence of the methylation of glycocyamine in the perfused liver *in situ*, though subcutaneous injection resulted in increased creatine excretion. Riesser[30] augmented the creatine content of rabbit muscle by injecting (with urea) choline and betaine; the administration of sarcosine with urea led to creatine formation in a number of experiments. Baumann and Hines[31] failed to obtain decisive results in perfusion experiments with sarcosine, betaine, choline, and methyl guanidine. Thomas and Goerne[32] obtained no creatinine formation in experiments with ϵ-guanido caproic acid and γ-methyl aminobutyric acid. Harding and Young[33] have reported that cystine administration intensified the creatinuria in young dogs. Recently, Gross and Steenbock[34] report that arginine given to pigs caused an increased creatine excretion; positive results obtained with cystine are explained by a resulting acidosis. From a study of the creatine elimination when sheep's thyroid was fed to pigs, they suggest that creatine formation is primarily dependent upon the balance that obtains between the arginase and the oxidative systems whereby arginine is destroyed; the thyroid principle accelerates the latter at the expense of the former.[35]

Before presenting the results obtained, we wish to explain that variations in the daily creatinine output in muscular dystrophy cases may be considerable, even with constant diet and care; Levene and Kristeller report a similar experience. The constancy

[24] Thompson, W. H., *J. Physiol.*, 1917, li, 111, 347.
[25] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 389.
[26] Inouye, K., *Z. physiol. Chem.*, 1912, lxxxi, 71.
[27] Doerner, G., *Z. physiol. Chem.*, 1907, lii, 225.
[28] Mellanby, E., *J. Physiol.*, 1908, xxxvi, 447.
[29] Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1917, xxxi, 549.
[30] Riesser, O., *Z. physiol. Chem.*, 1913, lxxxvi, 415; 1914, xc, 221.
[31] Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1918, xxxv, 75.
[32] Thomas, K., and Goerne, M. G. H., *Z. physiol. Chem.*, 1918–19, civ, 73.
[33] Harding, V. J., and Young, E. G., *J. Biol. Chem.*, 1920, xli, p xxxvi.
[34] Gross, E. G., and Steenbock, H., *J. Biol. Chem.*, 1921, xlvii, 33.
[35] Gross, E. G., and Steenbock, H., *J. Biol. Chem.*, 1921, xlvii, 45.

of the creatinine excretion cannot be the commonly accepted check on the collection of the day's specimen (*cf.* McCrudden and Sargent[36]) for such cases.

EXPERIMENTAL.

The experimental data which we present were obtained on one of our series of muscular atrophy cases (Case 7). We had hoped to confirm and extend these observations, but owing to more pressing clinical problems, it has been impossible to do so. A preliminary report of our findings has been made.[37] A brief description of the case follows:

W. Stil., white, male, 12 years of age, weight 30.5 kilos, entered the State University of Iowa Hospital on Sept. 5, 1919. The patient had not walked for 2 years, but the condition had been noted 2 years before that time. The diagnosis was progressive pseudohypertrophic muscular dystrophy. There was general muscular weakness and marked atrophy, especially of the deltoids. There were contractures of the hamstrings, toes, and hips. The calf muscles showed the characteristic pseudohypertrophy. The patient was observed for 3 months in the metabolism unit. At the time of his discharge from the hospital, he weighed 39 kilos. The metabolic findings are given in Table I.

Three low protein periods are given, days 2 to 8 inclusive, 26 to 34 inclusive, and 76 to 79 inclusive; the protein intake for the first two periods was 31.6 gm. and for the third 40 gm.; the energy value was 1,600 calories. Throughout the observations, the diet was non-purine and non-creatine. The diet for days 2 to 8 consisted of bread and honey with 250 gm. of milk. On the low protein diet, the total nitrogen elimination was about 3 gm., the creatinine from 0.12 to 0.15 gm., and the creatine a little over 0.3 gm. Ingested creatine is completely and promptly eliminated (days 5 and 28).

On a high protein régime (75 gm. of protein), the creatinine excretion was increased so that figures of 0.16 to 0.175 are the rule; the creatine elimination at times was over 0.5 gm. per day. The total nitrogen varied from 6.25 to 8.67 gm. The figures are for the periods on days 16 to 22 and 40 to 47. The creatine curve follows the urea nitrogen rather than the total nitrogen excre-

[36] McCrudden, F. H., and Sargent, C. S., *Arch. Int. Med.*, 1916, xvii, 465.
[37] Gibson, R. B., and Martin, F. T., *J. Biol. Chem.*, 1920, xli, p. xxxvi.

TABLE I.

Day.	Total N.	Urea N.	Creatinine.	Creatine.	Protein intake.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
2	2.97		0.151	0.312	31.6	
3	3.02		0.129	0.331	31.6	
4	2.49		0.121	0.331	31.6	
5	3.63		0.166	0.672	31.6	Creatine, 0.5 gm. by mouth.
6	3.32		0.140	0.445	31.6	
7	3.48		0.141	0.334	31.6	
8	2.92		0.141	0.289	31.6	
16	6.25		0.178	0.394	75.0	
17	6.45	4.98	0.162	0.487	74.0	
18	6.59	5.26	0.173	0.398	76.0	
19	7.24	5.82	0.177	0.465	75.0	Diet included 63.8 gm. of edestin.
20	7.74	4.06	0.136	0.342	77.0	Diet included 40.0 gm. of edestin.
21	7.50	4.92	0.176	0.484	76.0	
22	7.16	4.14	0.170	0.338	76.0	
26	3.98	2.20	0.144	0.295	32.0	
27	3.01	2.16	0.126	0.308	32.0	
28	4.21	3.04	0.160	0.782	31.0	Creatine, 0.6 gm. by mouth.
29	3.59	2.30	0.157	0.388	32.0	
30	3.17	2.21	0.144	0.310	32.0	
31	3.12	2.11	0.142	0.305	33.0	
32	3.15	2.30	0.142	0.312	32.0	
33	3.15	2.20	0.149	0.292	33.0	
34	3.25	2.08	0.150	0.317	33.0	Glucose, 55 gm. by mouth.
40	6.71	4.85	0.154	0.530	75.0	
41	7.32	5.04	0.173	0.510	75.0	
43	8.67	5.89	0.169	0.555	75.0	
44	7.55	4.45	0.168	0.457	75.0	
45	8.34	4.85	0.172	0.525	75.0	
46	8.11	6.45	0.170	0.493	75.0	Diet included 63.5 gm. of edestin.
47	7.55	5.78	0.157	0.448	75.0	
48	7.65	4.99	0.159	0.415	75.0	
49	8.18	6.02	0.159	0.477	75.0	
50	10.25	8.15	0.174	0.640	75.0	Diet included 50.3 gm. of gelatin.
51						Urine lost; diet as on day 50.
52	9.69	8.02	0.180	0.654	75.0	Diet included 55 gm. of gelatin.

TABLE I—*Concluded.*

Day.	Total N.	Urea N.	Creatinine.	Creatine.	Protein intake.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
53	8.86	7.24	0.179	0.615	75.0	Diet as on day 52.
54	7.04	5.63	0.162	0.560	75.0	
55	7.88	6.55	0.174	0.546	74.0	
56	7.69	5.94	0.168	0.525	75.0	Sarcosine, 1 gm. by mouth.
57	8.27	7.21	0.170	0.534	75.0	
58	7.46	6.06	0.168	0.552	75.0	Asparagine, 1 gm. by mouth.
59	7.60	6.20	0.162	0.560	75.0	
60	7.41	5.85	0.161	0.757	75.0	Glycocyamine, 0.5 gm. by mouth.
61	7.55	6.07	0.162	0.638	75.0	
62	9.60	7.46	0.162	0.610	136.0	Hordein, 61 gm. added to diet.
63	7.64	6.07	0.162	0.475	76.0	
66	8.58	6.13	0.155	0.478	75.0	
67	7.51	6.24	0.152	0.514	75.0	
68	11.00	8.82	0.190	0.689	75.0	
69	9.80	7.56	0.197	0.637	75.0	
70	8.19	5.67	0.151	0.417	75.0	
71	8.40	6.99	0.148	0.447	76.0	
74	5.46	3.87	0.147	0.372	40.0	
75	5.18	4.11	0.159	0.376	40.0	
76	4.69	3.32	0.153	0.298	40.0	
77	4.43	2.57	0.148	0.313	40.0	Sodium benzoate, 4 gm.
78	4.38	3.08	0.147	0.313	40.0	
79	3.99	2.44	0.147	0.306	40.0	
80			0.142	0.272	40.0	Histidine, 1.2 gm. by mouth.

tion. Replacement of 63.5 gm. of the protein of the diet by the arginine-rich protein edestin (days 19 and 46) failed to increase the creatine output over figures for some of the control days.

In order to increase the catabolism of the ingested protein, and try out the effect of feeding the incomplete protein gelatin, 50 to 55 gm. of gelatin were substituted for corresponding amounts of the mixed protein of the diet (days 49 to 53). Gelatin is practically creatine-free, commercial samples analyzed by Miss Booher in this laboratory contained 25 to 32 mg. of total creatinine per 100 gm. of gelatin of which only 1 to 6 mg. represent creatine. An increased elimination of total nitrogen (10.25 gm.),

urea nitrogen (8.15 gm.), creatinine (0.180 gm.), and creatine (0.654 gm.) resulted. Unfortunately, the specimen for day 51 was lost. The increased catabolism is to be regarded as exogenous, since Murlin has shown that nitrogen equilibrium can be maintained on a low level when two-thirds of the protein of the diet are replaced by gelatin. The progressive nitrogen retention (days 52 and 53) probably indicates an adjustment of the metabolism to utilize part of the amino-acids of the gelatin for growth purposes. Some days later (days 67 to 72), the patient for some unknown reason catabolized all of the ingested protein on 1 day and the metabolism was 2 more days in returning to the previous level. The metabolic picture is much like that obtained for the gelatin substitution experiment just presented.

In a further attempt to associate characteristically constituted proteins with creatine formation, hordein was given on 1 day; this protein yields no lysine on cleavage and is rich in proline. On account of possible deficient digestion and absorption of the prolamine protein preparations, 61 gm. of hordein were given in addition to the 75 gm. of the protein of the diet. In spite of an increase in the total and urea nitrogens, the creatine fell on this day and reached a low figure on the following day. The experiment is not satisfactory, however, as the creatine had not reached its customary level on account of the glycocyamine observation.

Sarcosine (day 56) and asparagine (day 58) were each without effect when given the patient. At least 36 per cent of 0.5 gm. of glycocyamine was converted into creatine without affecting the creatinine figure (day 60). Correction was made for the conversion of glycocyamine into glycocyamidine in the creatine analyses by a control determination after adding 50 mg. of glycocyamine to a tenth of the urine of a later day; 20 per cent of the glycocyamine was so converted. Assuming that glycocyamine might be a stage in creatine synthesis in the body from glycine and the guanidine nucleus, 4 gm. of sodium benzoate were given to divert part of the available glycine as hippuric acid (day 78). The results were negative. 1.2 gm. of histidine was given the patient on the last day that he was in the hospital. There was no effect on the creatinine or creatine excretion.

Confirmatory evidence as to the effect of high protein intakes on creatinine and creatine excretion has been obtained on other progressive pseudohypertrophic muscular dystrophy cases in our series. The substitution (or addition to the diet) of egg white protein has not indicated that the high cystine content is more effective in inducing creatine elimination. The administration of cystine has given negative results also.

CONCLUSIONS.

The following conclusions may be drawn from the data presented:

Ingested creatine was promptly and completely eliminated chiefly as creatine, in part as creatinine, in an advanced progressive pseudohypertrophic muscular dystrophy case.

The creatine and to a lesser extent the creatinine excretion was increased as the result of a greater protein intake.

This increase is obtained only from the protein that is catabolized, including gelatin, and not from that retained for growth purposes. Preformed creatine in the diet was not an important factor to be considered in interpreting the results.

The substitution of the arginine-rich protein edestin for 0.8 of the protein of the diet failed to increase the creatine excretion.

Hordein added to the diet increased the total nitrogen and urea elimination, but probably was without effect on the creatine; this observation is indicative only.

Ingested sarcosine and asparagine did not lead to an increase in the creatine excretion. Glycocyamine was converted in part (at least 36 per cent) into creatine. It is probably not a stage in ordinary creatine formation.

Experiments with cystine have been negative.

THE ACTION OF NITROUS ACID ON CASEIN.*

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INTRODUCTION.

The current theory of the nature of the free amino groups of the protein molecule holds that these consist largely of the epsilon amino groups of lysine. This assumption is based upon the evidence obtained by Van Slyke and Birchard (1) and others (2) who found that the nitrogen liberated from native proteins by the action of nitrous acid for 30 minutes was approximately one-half of the lysine nitrogen present. However, it was the view of Kossel and Gawrilow (3) that the existence of a quantitative relationship between the free amino nitrogen and the lysine content of a protein was improbable. Evidence in support of this view has recently been obtained by Edlbacher (4) and by Felix (5) who believe that there are free amino goups in the protein molecule other than those of lysine. These investigators observed that the lysine-free proteins, clupeine and salmine, methylated as easily as lysine-containing proteins and that the free amino nitrogen content of histones, sturines, gelatin, and glycinin was greatly in excess of one-half of the lysine nitrogen of these proteins. It has also been shown by Herzig (6) that deaminized gelatin is methylated as easily as gelatin itself. These observations would seem to establish the fact that there are groups in the protein

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† We wish to acknowledge our indebtedness to Professor Lafayette B. Mendel, of Yale University, who some years ago called the attention of the senior author (L) to certain aspects of Skraup's work.

molecule, other than the free amino groups of lysine, which are capable of methylation but it does not follow of necessity that all of the groups which have been methylated are free amino in character.

In view of the importance of this problem it seemed desirable to undertake a careful study of the product formed by the treatment of proteins with nitrous acid. Inasmuch as casein is easily obtained pure and its deaminaton product is insoluble, this protein was chosen for a study of the effect of deamination on the properties and composition of the protein molecule.

The Preparation of Deaminized Casein.

Pure casein was prepared according to the procedure of Van Slyke and Bosworth (7) omitting the treatment with ammonium oxalate.

Nitrous acid was first used as a deaminizing agent for proteins in 1885 by Loew (8). This investigator found that one-third of the nitrogen of peptones was liberated by the action of nitrous acid. In 1896 Paal (9) used silver nitrite and hydrochloric acid to deaminize gelatin peptones, while in the same year Schiff (10) obtained a straw-yellow compound by the interaction of nitrous acid and egg albumin. 2 years later Schrötter (11) observed the formation of a similar substance from peptones. In 1908 Treves and Salomone (12) allowed nitrous acid to react with egg albumin and obtained a yellow product which they believed was diazo albumin. Deaminized gelatin was obtained by Blasel and Matula (13) in 1914.

Skraup (14) and his pupils have prepared and studied the deaminized products of the following proteins: casein (15); gelatin (16); albumin (17); serum globulin (18); and edestin (19). Deaminized albumin, gelatin, and casein (20) and deaminized gliadin and vitellin (21) have been prepared and investigated by Levites.

Levites and Skraup have been largely responsible for perfecting the methods used in preparing deaminized protein products. In the first method of Levites (20) a paste was made from the protein and sodium nitrite, warmed on the water bath, treated with dilute acetic acid, and the resulting olive-green product dried *in vacuo*. In his second (21) procedure Levites produced an emulsion of the

protein by agitating it vigorously in a shaking machine with 10 per cent acetic acid. This emulsion was warmed gently on the water bath with a 10 per cent solution of sodium nitrite and the resulting yellow product filtered, washed with alcohol and ether, and dried *in vacuo*. In the method used by Skraup (15) an acid solution of the protein, prepared by adding glacial acetic acid to a uniform suspension of the protein in water, was warmed gently with sodium nitrite on the water bath and the yellow product drained off on linen, desiccated, and dried in air.

In the present series of experiments deaminized casein was prepared according to the methods outlined by Levites and Skraup. It was difficult to obtain a product of uniform color and appearance while the yield of 70 per cent reported by Skraup was rarely exceeded. There are several objections to the use of a shaking machine as employed by Levites. It requires several hours to emulsify small amounts of protein and the emulsified product almost invariably contains gelatinous lumps which must be ground up in a mortar to avoid contamination of the deaminized product with unchanged protein. Most of the methods used for the preparation of deaminized proteins employ heat up to 40°C. to effect complete deamination of the protein. It is possible that this application of heat may cause a slight hydrolysis and other change in the protein.

It is believed that the following procedure overcomes the objections cited above. 100 gm. of casein were added to 2 liters of distilled water contained in a 5 liter Pyrex flask. After stirring vigorously for 30 minutes with a mechanical stirrer a uniform suspension of the protein resulted. To this suspension 140 cc. of glacial acetic acid were added dropwise, with continued stirring, during the course of 1.5 hours. At the expiration of 20 minutes a good emulsion was formed, while at the end of the period, solution was effected. To this solution, 500 cc. of a solution of sodium nitrite containing 80 gm. to the liter were added dropwise, with continued stirring, during the period of 1.5 hours. After 150 cc. of this solution had been added a deep yellow precipitate rose to the top of the liquid as a yellow layer which, after standing for 18 hours, was filtered on a Buchner funnel using suction and a hardened filter paper. After triturating this substance fifteen times with hot water to the disappearance of an acid reaction to

litmus, it was granular and light yellow in color while the aqueous filtrate was similarly colored. The yellow precipitate obtained by triturating four times with 95 per cent alcohol was thoroughly desiccated by triturating three times with dry ether, drying in air for 30 minutes, and in the oven at 80°C. for an equal length of time. Although the alcoholic filtrate was highly colored the precipitate appeared to have lost but little of its yellow color in the washing process.

The deaminized product was of a uniform color and appearance and it was possible to secure practically a complete transformation into the deaminized form. From three 100 gm. samples of the original casein yields of 90, 95, and 97.5 gm. of the oven-dried products were obtained. These deaminized products, designated subsequently as deaminized caseins A-64, A-66, and A-68 were very fine and powdery when passed through an 80 mesh sieve.

With the second method of Levites, 17 gm. of deaminized casein A-18 were prepared from 25 gm. of casein. However, instead of permitting a complete deamination to occur the white precipitate formed upon the addition of the sodium nitrite solution was filtered on a Buchner funnel as soon as possible. This precipitate, which was triturated fourteen times with hot water, three times with 95 per cent alcohol, and twice with dry ether, was only faintly colored yellow.

The Properties of Deaminized Proteins.

Color.—Without exception, the deaminized protein products cited in the literature are yellow in color. The products prepared in the present research were colored light yellow when first prepared, but surfaces exposed to the light for a time became light brown. Treves and Salomone (12) believed that these substances were diazo derivatives since they responded to the reactions given by diazo compounds. It is known, however, that primary aliphatic amines do not react with nitrous acid under ordinary conditions to give stable diazo derivatives. Furthermore were this a reaction with the amide groups which are present acids would be produced (22) and not diazo products. Therefore the assumption of these authors seems untenable.

It is possible that deaminized proteins are colored because of the formation of nitroso compounds. There are numerous possi-

bilities for nitrosation in the protein molecule. Histidine, tryptophane, and proline, each have one imino nitrogen, while there are two such nitrogens in the guanidine group of arginine. A nitroso group might enter tyrosine in the position ortho to the hydroxyl group in the benzene ring. It is unlikely that a nitrosation of the imide nitrogen making up the peptide linkage has taken place although it has recently been shown (23) that the nitroso derivative of methyl phthalimidine is easily formed by treatment with nitrous acid in water solution. Since the carbonyl imide linkage present in methyl phthalimidine is the same as that found in peptides a possible nitrosation of the peptide linkage is suggested. However, this cannot have taken place to any marked extent because deaminized proteins have been shown to contain less nitrogen than the original proteins.

Solubility.—Deaminized proteins are reported to be insoluble in water and insoluble or only slightly soluble in alkalies. Skraup noted the formation of a jelly-like substance when deaminized proteins were brought into contact with strong alkali. It was found in the present investigation that deaminized casein dissolved in 0.5 per cent sodium hydroxide after standing for 2 days with the formation of a red solution and a small amount of undissolved residuum. With 1.5 per cent sodium hydroxide solution a red solution was formed in 24 hours while with concentrated alkali an orange to brown jelly was formed in a few minutes.

Color Reactions.—Deaminized casein prepared according to the method described above gave positive tests with Hopkins-Cole, Millon's, and biuret reagents. Millon's test was unquestionably positive although the color was less intense than with casein but the biuret reaction with deaminized casein was not characteristic ranging from a pink to a reddish purple. Levites (20) is the only investigator to report a positive Millon's reaction with deaminized proteins while the biuret reaction was found to be positive by Levites (20) and by Treves and Salomone (12). If it be true that deaminized proteins give a positive biuret reaction this would indicate that the grouping which is responsible for the color is not attacked or at least is only partially destroyed by the action of nitrous acid.

Composition.—The elementary composition of native proteins appears to be but little altered in the deamination process. Skraup

(14) found a slight diminution in the phosphorous content of deaminized casein and a constant increase, with one exception, in the oxygen content of all of the deaminized proteins studied but neither observation was considered to be of particular significance. It is striking, however, that the nitrogen content of deaminized proteins is lower than that of the original substance. Schiff (10) reported a reduction of 1.0 per cent in the values for nitrogen while the figures quoted by Skraup (14) range from 0.51 to 1.24 per cent lower in nitrogen than those of the original proteins. In the case of edestin (19) for some unaccountable reason the nitrogen of the deaminized product was found to be higher than that of the original protein.

It will be noted from Table I that the nitrogen content of the deaminized casein prepared in this research ranges for the various samples from 0.22 to 0.68 per cent lower than the figures obtained for the original casein.

TABLE I.

Sample.	Nitrogen calculated on an ash-free basis.
	per cent
Casein 1	14.56
Deaminized casein A-18	13.91
" " A-64	14.34
" " A-66	13.88
" " A-68	14.01

Free Amino Nitrogen.—In the present study of deaminized casein, free amino nitrogen was determined according to the method outlined by Van Slyke (1) with the use of the micro apparatus. To a suspension of 3 gm. of casein in distilled water was added a solution containing 0.375 gm. of sodium carbonate. The casein, which was usually in complete solution within an hour, was transferred to a 100 cc. volumetric flask, diluted to the mark, and 2 cc. were taken for amino nitrogen analysis. This solution is neutral to litmus and as Van Slyke (1) has shown, an inappreciable hydrolysis takes place even after standing at room temperature for 48 hours. In the deamination of casein a foam inhibitor was found to be indispensable. For this purpose caprylic alcohol was found

to be more effective than diphenyl ether although the blank resulting from the former substance was, as has been reported (24), considerably higher than that given by diphenyl ether. Even without the caprylic alcohol the blank from the sodium nitrite was in general slightly higher than that reported by Van Slyke for good samples of this substance. Casein was found to precipitate from solution immediately upon contact with the acid solution in the deaminizing chamber and to gradually change from a pure white to a yellow. Because of the fact that casein must undergo

TABLE II.

The Free Amino Nitrogen Content of Casein and Deaminized Casein.

Samples.	Total nitrogen as free amino nitrogen.
	per cent
Casein, Kahlbaum	5.61
" " after Hammarsten	5.99
" after Van Slyke and Bosworth (7)	5.68
" " Van Slyke and Baker (28)	5.52
" Osborne* No. 1	6.13
" " " 2	5.84
" " " 3	6.24
" " " 4	6.22
" " " 5	6.04
" " " 6	5.47
Deaminized casein A-18	3.10
" " A-64	0.00

* Casein samples Nos. 1 to 6 were secured through the kindness of Dr. T. B. Osborne of the Connecticut Agricultural Experiment Station, New Haven, Connecticut. In every case unusual procedures were used in the preparation of these samples.

deamination in the solid state reliable results are to be obtained only by maintaining constant conditions. Uniform results were secured by shaking the deaminizing chamber for 30 minutes at 300 vibrations per minute.

Van Slyke (1) has reported that the free amino nitrogen of casein comprises 5.51 per cent of its total nitrogen content. Since there are various methods which are used for the preparation of casein from cow's milk it was considered of importance to determine the free amino nitrogen of samples of casein prepared in a

TABLE III.

Time of hydrolysis.	Tyrosine.	
	Casein 1.	Deaminized casein A-64.
hrs.	*per cent*	*per cent*
12	5.56	3.88
16	5.98	4.03
19	5.78	3.57
Average..............	5.77	3.82

variety of ways on the assumption that the reagents employed in the purification of this protein might possibly have altered its free amino groups. However, the results which were obtained (Table II) were not widely divergent and it would seem that the reagents employed in the preparation of these samples of casein were without appreciable influence upon the number of free amino groups of this protein. The average figure for the free amino nitrogen content of ten samples of casein was found to be 5.87 per cent.

Casein A-18, which was only slightly yellow in color was prepared by treatment with nitrous acid but was removed from the influence of this reagent as soon as possible after precipitation.

TABLE IV.

*The Distribution of Nitrogen in Casein and Deaminized Casein.**

Nitrogen.	Casein nitrogen.		Deaminized casein nitrogen.	
	gm.	*per cent*	*gm.*	*per cent*
Amide............................	0.0916	10.49	0.0921	11.09
Humin............................	0.0190	2.13	0.0237	2.85
Arginine..........................	0.0673	7.42	0.0588	7.09
Histidine.........................	0.0537	6.01	0.0323	3.89
Lysine............................	0.0812	9.09	0.0056	0.67
Cystine...........................	0.0043	0.48	0.0022	0.26
Monoamino.........................	0.5253	58.78	0.5520	66.50
Non-amino.........................	0.0530	5.93	0.0486	5.85
Sum...............................	0.8954	100.33	0.8153	98.20
Total nitrogen by Kjeldahl........	0.8935	100.00	0.8300	100.00

* The figures in each case represent the average of duplicate determinations.

The color reactions given by this product were unquestionably positive and quite comparable to the reactions given by casein itself. As is given in Table II, the free amino nitrogen of this product was 3.10 per cent of the total nitrogen while that of completely deaminized casein was 0.0. Since the value for casein A-18 lies midway between that given by unchanged casein on one hand and completely deaminized casein on the other it would seem that at the time of precipitation only partial deamination had occurred.

Total Amino Nitrogen.—The conditions necessary for the complete hydrolysis of proteins have been determined. Comparable results have been secured by heating in an autoclave at 150°C. for 1.5 hours with 3.0 N hydrochloric acid (25) and by boiling at 100°C. for 24 to 48 hours with 20 per cent hydrochloric acid (26). With either of these methods the maximum amount of amino nitrogen is liberated from peptide linkage but according to Van Slyke there is less tendency towards deamination of amino-acids, particularly cystine (27), at 100°C. than at 150 or 160°C.

Complete hydrolysis of 5 gm. samples of casein and deaminized casein was effected by autoclaving for 3 hours at 124°C. with 200 cc. of 3.0 N hydrochloric acid. Henriques and Gjaldbäk (25) found that under these conditions the results of hydrolysis were approximately the same as those obtained by heating for 1.5 hours at the higher temperature. In both cases some undissolved particles remained after autoclaving and the supernatant liquid of the deaminized casein was colored a deeper brown than that obtained from casein. Each hydrolysate was evaporated to dryness on the water bath, taken up with water, and diluted to the mark in a 500 cc. volumetric flask. Amino nitrogen analyses were made on 1 cc. portions of the hydrolysates by means of the Van Slyke apparatus.

The total amino nitrogen of casein was found to be 69.1 per cent of its total nitrogen content while 68.5 per cent of the total nitrogen of deaminized casein was present in the amino form. Since the difference observed between these percentages is probably within the range of experimental error, it is believed that the total amino nitrogen of casein and deaminized casein is the same.

The Tyrosine Content of Deaminized Casein.

Despite the fact that some investigators have obtained a positive Millon's reaction with deaminized proteins the statement has been made that "Von den Monoaminosäuren ist zu sagen, dass ihr Gehalt quantitativ wahrscheinlich unverändert bleibt, mit Ausnahme des Tyrosins, das bei allen Desamidoproteinen fehlt" (21). This conclusion is based largely upon the work of Skraup who could not obtain crystalline tyrosine from deaminized proteins although other monoamino acids were isolated in amounts comparable to those present in the original protein. Since deaminized casein was found to give a positive Millon's test in the present study, the presence of tyrosine would seem to be indicated. That this assumption was correct was definitely proved by the following experiment. 10 gm. samples of casein and deaminized casein were hydrolyzed for 12 hours with 200 cc. of 20 per cent sulfuric acid and tyrosine isolated in both cases by the usual procedure. Tyrosine was identified by its crystalline form and by its color reactions with Millon's and Mörner's reagents. Although no effort was made to obtain quantitative yields the amount of tyrosine obtained from deaminized casein appeared to be only slightly less than that obtained from casein.

The quantitative determination of tyrosine in casein and deaminized casein was made according to the colorimetric method of Folin and Denis (29). Although Abderhalden (30) and Gortner (31) have shown that the blue color given by the phenol reagent is not specific for tyrosine, Johns and Jones (32) have observed that the blue color with tryptophane is less intense than that given by an equivalent amount of tyrosine while decomposition products of tryptophane give no color at all. Therefore these authors concluded that reliable results for tyrosine in proteins can be obtained with Folin's colorimetric method.

According to the results obtained in the present study (Table III) the average figure for the tyrosine content of casein, 5.77 per cent, is 0.73 per cent lower than the value, 6.50 per cent, found by Folin. Johns and Jones (32) believe that some tyrosine is destroyed if acid hydrolysis is continued longer than 12 hours, but the data obtained in the present study do not support this contention. It is evident, however, that some of the tyrosine or the substances reacting with the Folin reagent

have been destroyed during the process of deamination since the tyrosine content of deaminized casein was found to be only about 66 per cent of that of casein. Since the deaminized casein products investigated by Skraup were subjected in their preparation to the action of heat up to 40°C. it is possible that tyrosine may have been destroyed or altered under these conditions. In this connection it is of interest to note that Levites obtained a positive Millon's test with desaminocasein (20, 21) prepared by his earlier method in which the protein was not subjected to heat in the presence of acetic acid, while no Millon's reaction was observed with desaminocasein (21) in the preparation of which the deamination took place in the presence of acetic acid and at 40°.

The Distribution of Nitrogen in Casein and Deaminized Casein.

Although in general the Van Slyke (33) procedure for the characterization of proteins was followed in the present investigation of the distribution of nitrogen in casein and deaminized casein, certain modifications were incorporated from unpublished results of Hamilton, Nevens, and Grindley. In each case, 6 gm. of casein and deaminized casein were taken for duplicate analyses and these samples were hydrolyzed in the usual manner. It was considered to be advantageous to distill off the excess hydrochloric acid *in vacuo* before removing an aliquot portion for the estimation of total nitrogen. By the use of a dual system for vacuum distillation duplicate determinations were made under exactly the same conditions of pressure. The apparatus for arginine, as modified by Hamilton, Nevens, and Grindley, permits the passage of purified air through the system during the entire 6 hours. Air under pressure is passed through a purifying train of concentrated sulfuric acid and concentrated alkali to remove carbon dioxide and ammonia and thence to the bottom of the Kjeldahl flask by means of a capillary glass tube inserted in the stopper of the Kjeldahl flask. The solution is boiled gently for exactly 5.5 hours and the water then drained from the condensers and the boiling continued for a half hour longer. It was found that if a bubble of air per second is allowed to escape from the Folin bulb that the last traces of ammonia are aerated into the standard acid and subsequent distillation is unnecessary.

The figures obtained for the distribution of nitrogen in casein and deaminized casein are given in Table IV. The analysis for casein agrees rather closely with the figures quoted in the literature and in general the figures obtained for casein and deaminized casein agree closely. Skraup (15) has reported a diminution in the percentage of arginine and histidine in deaminized casein and the entire absence of lysine in the deaminized product. In the present research the figure for arginine nitrogen in deaminized casein, 7.09 per cent, is only 0.33 per cent lower than the figure in casein, 7.42 per cent. It is believed that this difference is within experimental error and does not signify a destruction of this amino-acid in the deaminized product. This is what would be expected if nitrous acid is without action upon the guanidine group of arginine which has been shown to be free in the protein molecule. If on the other hand, it is true as has recently been reported by Sekine (34) that the guanidine group of arginine is slowly attacked within the space of a few hours by nitrous acid at low temperatures some destruction of arginine would be expected. Although the value for the histidine nitrogen in deaminized casein, 3.89 per cent, is less than the figure found for casein, 6.01 per cent, it must be borne in mind that by this method histidine is not determined directly. Hence, although these results indicate a partial destruction of histidine, a final conclusion as to the content of histidine in deaminized casein is not warranted. The figure for lysine nitrogen in casein was found to be 9.09 per cent while in the deaminized product 0.67 per cent was obtained. Since lysine is determined by indirect calculation, the latter percentage is probably within experimental error and it is considered as probable that no lysine is present in deaminized casein. It would seem, therefore, that this observation is in harmony with the work of Skraup, who could isolate no lysine from the hydrolyzed products of deaminized proteins and with the current theory concerning the nature of the free amino groups of the protein molecule. The belief that the free amino nitrogen of native proteins is due at least in part to the epsilon amino group of lysine renders likely the assumption that deamination of native proteins cleaves the terminal amino group of lysine to form α-amino-ϵ-hydroxy caproic acid, or some similar derviative. Since this derivative of lysine would not be basic its subsequent reactions would be those common to the

monoamino-acids; *i.e.*, it would not be precipitated by phosphotungstic acid but would appear in the monoamino filtrate. The marked increase in monoamino nitrogen in the filtrate indicates that this or some similar derivative has become associated with the mono-acid fraction. Attempts made by Skraup and others to isolate this hydroxy derivative of lysine have failed. Skraup (16) claims to have obtained the anhydride of α-amino-δ-hydroxy valerianic acid which he believes has been formed from the hydroxy caproic acid derivative of lysine. These results are similar to those obtained by Levites (21) with the older method of analysis. He obtained 10.05 and 10.36 per cent of amide nitrogen, 69.00 and 81.69 per cent of monoamino nitrogen, and 20.03 and 7.20 per cent of diamino nitrogen in casein and desaminocasein, respectively. The desaminocasein which he analyzed had been heated to 40° in the presence of acetic acid.

In the present investigation it seemed likely from direct determinations of tyrosine in the original acid hydrolysates (see Table III) that some of this amino-acid had been destroyed in the process of deamination. To obtain further evidence upon this point analyses of tyrosine in the monoamino-acid filtrates of casein and deaminized casein were made. In the case of casein, 5.41 and 5.89 per cent were found while 2.72 and 2.98 per cent of tyrosine were found in the monoamino-acid filtrates of deaminized casein. It is evident, therefore, that a considerable part, about 50 per cent of the tyrosine or the substance which gives the blue color with the phenol reagent, has been destroyed during the process of deamination.

SUMMARY.

1. The free amino nitrogen of a number of samples of casein, prepared in various ways, has been determined and found to be nearly constant.

2. A modification of the method of Skraup for the deamination of casein has been described which yields larger quantities of the deaminized product.

3 Tyrosine was isolated from the hydrolysis products of deaminized casein. Quantitative determinations of tyrosine in casein and deaminized casein showed that tyrosine was partially de-

stroyed in the process of deamination but not completely so as maintained by Skraup.

4. The distribution of nitrogen in casein and deaminized casein has been determined by the Van Slyke partition method. In harmony with the current theory as to the nature of the free amino groups of the protein molecule, deaminized casein was found to contain no lysine. The monoamino nitrogen of the filtrate of deaminized casein was increased, the increase being nearly proportional to the decrease in lysine nitrogen. No other notable differences between casein and deaminized casein were found.

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A COMPARATIVE STUDY OF THE HYDROLYSIS OF CASEIN AND DEAMINIZED CASEIN BY PROTEOLYTIC ENZYMES.*

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The observations reported in the literature in regard to the digestibility of deaminized proteins are conflicting. Treves and Salomone (1) reported that deaminized proteins are not digested by artificial gastric or pancreatic juice while Schiff (2) found them to be completely digested by dog's gastric juice although the rate of digestion was much slower than with the original proteins. Levites (3) found digestion to be complete with dog's gastric juice.

In a review of the methods used for studying digestion *in vitro* the statement is made by Frankel (4) that "the best index of the extent to which a protein has been disintegrated is the ratio of the amino nitrogen at a given time to the total amino nitrogen obtained after complete hydrolysis." Of the various methods by which the amino nitrogen of proteins may be estimated Frankel chose the Van Slyke procedure as "best suited to the problem in hand." After the examination of various methods for the study of proteolytic action Sherman and Neun (5) concluded that "the quantitative determination of . . . . the amino nitrogen of the digestion products . . . . appears to be more delicate as a means of detecting proteolysis than either the biuret or the ninhydrin reaction and more delicate, accurate, and generally applicable as a means for its measurement than any of the other quantitative methods here studied." These authors used the

* An abstract of a thesis submitted by Max S. Dunn in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in the Graduate School of the University of Illinois.

Van Slyke method for estimating amino nitrogen but assumed that results with the Sörenson method would run parallel.

In the proteolytic studies of the present investigation the action of pepsin, trypsin, and erepsin alone and in series was studied. The samples of pepsin and trypsin used in these experiments were commercial preparations known to be active while erepsin was prepared from the intestinal mucosa of a dog according to Frankel's (4) modification of the method outlined by Rice (6). The erepsin preparation was considered to be trypsin-free since it was without action upon fibrin. For the determination of the total amino nitrogen available on complete hydrolysis, samples of casein and deaminized casein were hydrolyzed according to the method of Henriques and Gjaldbäk (7), but the total amino nitrogen was determined by the nitrous acid method of Van Slyke instead of by formol titration. From this figure, the free amino nitrogen of the intact protein molecule was subtracted and the resulting figure was considered to represent the amino nitrogen in peptide linkage; *i.e.*, the maximum amount of amino nitrogen actually available for liberation by enzymes. The liberation of amino nitrogen during digestion was followed by means of the Van Slyke micro apparatus. Frankel ran controls "with all reagents and ferments in the same quantities except that no protein was added" to correct for the amino nitrogen present in the reagents. In the present study control experiments were carried out with the same amounts of protein and reagents, but using boiled enzyme solutions. By this technique corrections are made not only for the amino nitrogen present in the reagents and enzyme added but also for the free amino nitrogen content of the proteins and for that which may have been liberated by the hydrolytic action of the reagents. It is believed, therefore, that the corrected values for the free amino nitrogen are an accurate measure of the amino groups actually liberated by the digestive action of the enzymes employed.

The results given in Table I and Chart 1 were obtained by the simultaneous digestion of 5.0 gm. samples of casein 1 and deaminized casein, A-64, the preparations of which were described in the preceding paper. These proteins were suspended uniformly in 250 cc. of 0.2 per cent hydrochloric acid and 20 cc. of an aqueous solution, containing 0.2 gm. of pepsin, added. Controls contain-

TABLE I.

The Peptic, Tryptic, and Ereptic Digestion of Casein 1 and Deaminized Casein A-64.

Enzyme.	Hours.	Total available amino nitrogen liberated.	
		Casein 1.	Casein A-64.
		per cent	*per cent*
Pepsin	0.0	0.0	0.0
"	14.5	9.6	2.6
"	38.5	11.1	3.3
"	86.5	11.7	3.3
"	110.5	11.8	3.3
Trypsin added at the end of 110.5 hours.			
Trypsin	122.5	53.2	25.8
"	146.5	68.3	30.9
"	170.5	79.9	33.4
"	242.5	78.7	33.5
Erepsin added at the end of 242.5 hours.			
Erepsin	260.0	91.7	56.2
"	284.0	92.1	65.4
"	308.0	95.8	65.8
"	332.0	95.7	65.4

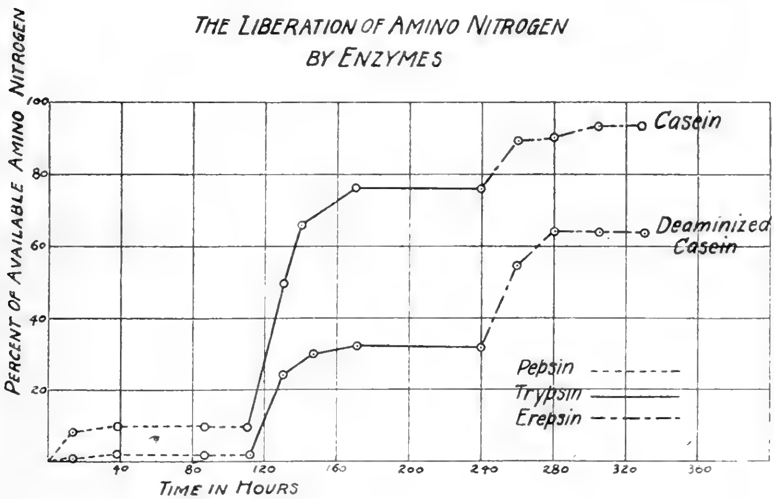

CHART 1.

ing the protein, all reagents, and boiled enzyme solutions were run. After thoroughly mixing the contents of each flask 5 cc. of toluene were added as a preservative and incubation at 38°C. was begun. At the end of 3 hours incubation the casein sample in which the active enzyme was present had gone entirely into solution, but the deaminized casein and the controls had settled out. The supernatant liquid of the deaminized casein sample was colored yellow indicating that a partial digestion had taken place since the supernatant liquid of the controls was colorless. At the expiration of the 3 hour period uniform samples from each flask were taken for analysis of amino nitrogen in the Van Slyke micro apparatus. To 5 cc. portions from each flask was added 0.5 cc. of N sodium hydroxide solution to stop digestion. The resulting solution was diluted to 10 cc. on a volumetric flask and 2 cc. were taken for analysis. At indicated intervals in the digestion subsequent amino nitrogen determinations were made. At the end of 110 hours digestion 200 cc. aliquots from each peptic digest were taken, 20 cc. of 15 per cent sodium carbonate solution and 0.4 gm. of trypsin added, and incubation at 38°C. was begun. Boiled trypsin solution was added to the controls. After several hours the deaminized casein sample went into solution giving a brown but transparent liquid but there appeared to be no change in the controls. At the end of 132 hours digestion with trypsin, 150 cc. aliquot portions were removed from each flask, 20 cc. of erepsin solution added, and incubation was continued for 89.5 hours. Boiled solutions of the enzyme were added to the controls. For the determination of amino nitrogen in the tryptic and ereptic solutions 5 cc. aliquot portions were used. After arresting digestion by the addition of 0.5 cc. of glacial acetic acid these samples were diluted to 10 cc. in a volumetric flask and 2 cc. used for the analysis of amino nitrogen.

Similar experiments were carried out with trypsin (Table II) and with erepsin (Table III) to determine whether these enzymes could attack deaminized casein without the preliminary action of pepsin. 2 gm. samples of casein 1 and deaminized casein A-64 were dissolved in 120 cc. of 0.5 per cent sodium carbonate solution. Casein 1 gave a solution of medium opalescence while casein A-64 formed a deep red solution in which gelatinous particles were suspended. 20 cc. of a solution containing 0.2 gm. of trypsin

dissolved in a 0.5 per cent solution of sodium carbonate or 20 cc. of the erepsin preparation were added to each flask. 10 cc. of an alcoholic solution of thymol were added in each case as a preservative. After incubation at 38°C. for 22.5 hours a 5 cc. sample from each flask was taken, 0.5 cc. of glacial acetic acid added to arrest digestion, and the resulting mixture diluted to 10 cc. in a volumetric flask. A clear but brown liquid in the case of casein A-64 indicated that some digestion had taken place in each instance. The appearance of the proteins in the control flasks was not altered.

The results obtained from the enzymatic hydrolyses of casein (Table I) agreed closely with the observations of Frankel (4). Pepsin liberated 11 per cent of the total available amino nitrogen of casein in 87 hours; trypsin superimposed upon the pepsin digest set free 79 per cent in 60 hours; and by the further action of erepsin for 66 hours 95 per cent was liberated. These values are maximum for these enzymes since the amino nitrogen was found to remain approximately constant over a period of 25 or more hours of additional digestion. The digestion of deaminized casein proceeded in every case at a slower rate than that of casein and the total cleavage was considerably less. Only 3 per cent of the total available amino nitrogen of deaminized casein was liberated after 110 hours of peptic digestion in contrast to 11 per cent with casein. Tryptic digestion for 132 additional hours set free only 33 per cent of the total available amino nitrogen as compared with 79 per cent for casein while the further action of erepsin liberated only 65 per cent as contrasted with 95 per cent for casein.

It is possible that difference in digestive action between casein and deaminized casein may be due to decreased solubility of the latter and its subsequent less intimate contact with the enzymes. There is also the possibility that reactions incidental to the process of deamination may have taken place to alter the peptide linkage in such a way that its cleavage by enzymes became more difficult.

It is evident from the results reported in Table II that trypsin will digest deaminized casein without the preliminary action of pepsin. However, the rate of digestion by trypsin alone is slower than that by trypsin after the preliminary action of pepsin, and the percentage of hydrolysis is less. The digestion of deaminized

TABLE II.

The Tryptic Digestion of Casein 1 and Deaminized Casein A-64.

Hours.	Total available amino nitrogen liberated.	
	Casein 1.	Casein A-64.
	per cent	*per cent*
0.0	0.0	0.0
22.5	41.3	25.3
45.0	50.4	27.9
68.5	52.7	27.8
92.5	52.4	28.7

TABLE III.

The Ereptic Digestion of Casein 1 and Deaminized Casein A-64.

Hours.	Total available amino nitrogen liberated.	
	Casein 1.	Casein A-64.
	per cent	*per cent*
0.0	0.0	0.0
21.5	8.0	0.0
45.5	8.0	0.0
93.5	11.6	0.0

TABLE IV.

Date.	Weight.	Total nitrogen.	Urea and ammonia nitrogen.	
1921	*kg.*	*gm.*	*gm.*	*per cent*
Jan. 7				
" 8				
" 9				
" 10	9.90			
" 11	9.90	1.355	0.841	62
" 12	9.88	1.342	0.910	67
" 13	9.88	1.251	0.757	60
" 14	9.86	1.427	0.872	61
" 15*	9.82	1.955	1.622	77
" 16	9.75	1.363	0.946	69
" 17	9.75	1.246	0.960	77
" 18	9.75	1.299	0.968	74
" 19	9.73			

* 10 gm. of deaminized casein A-64 were fed in addition to the standard diet.

casein by trypsin is less extensive and proceeds at a slower rate than that of casein.

Since it is known that casein is attacked by erepsin without the preliminary action of other enzymes it was of interest to test the action of this enzyme towards deaminized casein. It was found (see Table III) that 11 per cent of the total available amino nitrogen of casein was liberated after 93 hours of ereptic digestion but no amino nitrogen was liberated from deaminized casein. It would appear, therefore, that erepsin does not attack deaminized casein easily.

Since in the present investigation digestion experiments *in vitro* indicated that deaminized casein was digested, although at a slower rate than casein, it was desirable to study the behavior of deaminized casein in the animal organism. A female dog, weighing about 10 kilos, was maintained upon a uniform diet for 10 days to permit a constant level of nitrogen excretion to be reached. At the expiration of the period 10 gm. of deaminized casein A-64 were added to the standard diet and the elimination of extra nitrogen in the urine determined.

The standard diet consisting of 400 cc. of water, 10 gm. of bone ash, 25 gm. of starch, 25 gm. of lard, 35 gm. of sucrose, and 50 gm. of beef heart was considered to be calorifically adequate for a dog of the size used. The urine was collected daily by catheterization. As shown in Table IV about 43 per cent of the added nitrogen was eliminated in the urine. Since there was a corresponding or slightly greater increase in urea nitrogen elimination the assumption that utilization of the deaminized casein in the animal organism has occurred seems well founded.

Further experiments were planned to include a study of the relative efficiency of casein and deaminized casein in the maintenance of nitrogenous equilibrium in the dog, but it was found that repeated administration of deaminized casein resulted in vomiting and loss of appetite. For this reason the experiments along this line were not carried further.

SUMMARY.

Casein and deaminized casein were digested *in vitro* by pepsin and trypsin. Erepsin digested casein readily but attacked deaminized casein only after the preliminary action of pepsin or

trypsin. In every case the digestion of deaminized casein proceeded at a slower rate than the digestion of casein. In one experiment after the feeding of deaminized casein to a dog the increased elimination of total and urea nitrogen indicated that the deaminized product was digested and metabolized in the animal body.

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NOTE ON THE DETERMINATION OF β-HYDROXYBUTYRIC ACID.*

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In studying the determination of β-hydroxybutyric acid three methods have been most used. These are: first, the isolation of the acid, with its subsequent determination by the polariscope; second, the dehydration of the acid to give α-crotonic acid; and third, the oxidation of the acid to give acetone.

In Hurtley's (1915–16) paper, in which the discovery and subsequent investigation of the properties of this acid are discussed exhaustively, it is shown that all of these methods are closely connected with the first studies made by Kulz (1884), Stadelmann (1883), and Minkowski (1884) into the properties of the organic acid isolated by them from urines which contained an excess of base greater than that theoretically needed to neutralize the inorganic acids found.

Kulz (1884) first fermented diabetic urine and determined the rotation, thus measuring the amount of the acid present. Wolpe (1886) modified this method. He extracted the acidified urine with ether, took up the extracted acid with water, and determined the acid in the extract by the polariscope. Magnus-Levy (1901), Bergell (1901), Geelmuyden, and Black (1908–09) improved this extraction method further. Ohlsson (1916) has recommended ethyl acetate instead of ether for the extraction.

The second method was suggested by Darmstaedter (1903). He converted β-hydroxybutyric acid into α-crotonic acid by the action of concentrated sulfuric acid, and determined the unsatu-

* This paper formed a part of a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Washington University, Saint Louis, in June, 1921.

rated acid by titration after distillation—a reaction noted by Kulz (1884) and Stadelmann (1883). This method has been modified by Pribram (1911–12) and others, but has not been found to be very satisfactory (Shaffer, 1908–09; Shindo, 1907).

The third method for the determination of β-hydroxybutyric acid was proposed by Shaffer (1908–09) based on a reaction mentioned by Minkowski (1884). β-Hydroxybutyric acid when oxidized with sulfuric acid and potassium dichromate forms acetone and carbon dioxide, probably giving acetoacetic acid as an intermediary product. In his first paper, Shaffer (1908–09) stated that this oxidation was quantitative. Embden and Schmitz (1910) in Abderhalden's Handbuch stated their belief that the results by this method were low. In 1913, Shaffer and Marriott (1913–14) studied the reaction, using pure β-hydroxybutyric acid prepared from calcium zinc β-hydroxybutyrate. They found that their recovery was from 5 to 10 per cent low. The method described is a slow one, as 3 to 4 hours are required for maximum oxidation with the amounts of sulfuric acid and potassium dichromate recommended. Marriott (1914), Folin and Denis (1914), and Kennaway (1914) have used this oxidation of β-hydroxybutyric acid in connection with different methods for the determination of acetone. Lately Van Slyke (1917) has studied the reaction carefully and investigated the causes that lead to incomplete oxidation. At the time the experiments reported below were performed, only the preliminary report of this last paper was available.

For determining β-hydroxybutyric acid in urine the method of oxidizing with acid and potassium dichromate was chosen as most suitable. Two main problems presented themselves for solution in using this method. Was it possible to find some way in which the determination could be made exact? That is to say, could conditions of the determination be found that would lead to a recovery of the theoretical amount of acetone from a given amount of β-hydroxybutyric acid? Could the time of oxidation be shortened? Many different oxidizing mixtures with widely varying amounts of sulfuric acid and potassium dichromate were tried, and large and small amounts of β-hydroxybutyric acid were used in the determinations, but a recovery of the theoretical amount of acetone was not accomplished. In attempting to

solve the second problem, an investigation of conditions governing the oxidation of β-hydroxybutyric acid by acid dichromate solutions was undertaken, and it was found that the rate and completeness of oxidation depend on the relative concentrations of acid and dichromate. Van Slyke (1917) has published a series of experiments establishing this fact, and the results found do not differ from those described by him.

Shaffer and Marriott (1913–14) give the following description of the method (p. 271): "the contents of the distilling flask containing the oxybutyric acid was diluted to about 600 cc., 30 cc. of sulphuric acid (sp. gr. 1.59) added, and a total of about 0.5 gram of $K_2Cr_2O_7$ in very dilute solution dropped in during the distillation which was continued about three and one-half hours."

Using this method as a basis, two methods were worked out for the determination of β-hydroxybutyric acid in a shorter period of time. The first, as applied to large amounts of the acid, has been described in Folin's Manual (Folin, 1916), and by Shaffer and Hubbard (1916). In this method β-hydroxybutyric acid was oxidized in the presence of 9 to 10 N sulfuric acid, and the amount of potassium dichromate was adjusted so that the reaction was complete in 15 minutes.

Continued use of this method showed that there were two objections to it; first, the acid was so strong that the tin tubes of the ordinary Kjeldahl still were quickly corroded; and second, when the technique was extended to very small amounts of acetone it was necessary to use a correspondingly decreased amount of potassium dichromate.

In working with conditions in which less sulfuric acid was used, a great many different procedures were tried out to find which one would give the maximum recovery in a short time. It was found impossible to get complete oxidation in 15 minutes, and the time was lengthened to half an hour, and potassium dichromate was added at intervals instead of by drops. If all the reagent was added at once, yields were lower.

After many trials of different amounts of acid and potassium dichromate, and of different methods of adding the dichromate, the following was selected: to the β-hydroxybutyric acid contained in 100 cc. of solution, heated to boiling in a Kjeldahl flask attached to a water-cooled condenser, 30 cc. of sulfuric acid

(concentrated sulfuric acid diluted with an equal volume of water) and 20 cc. of a potassium dichromate solution, 0.1 to 0.2 per cent, were added through a dropping funnel. The burners were regulated so that about 50 cc. distilled in 10 minutes. After 10 minutes 50 cc. of 0.1 to 0.2 per cent potassium dichromate were added and the distillation was continued; 10 minutes later 50 cc. more of 0.1 to 0.2 per cent potassium dichromate were added and distillation was continued another 10 minutes. The boiling was not interrupted while the additions were made. The total distillate

TABLE I.

Determination of Pure Solutions of β-Hydroxybutyric Acid.

Oxidized for $\frac{1}{2}$ hour as described (results are expressed in terms of acetone).

Present.	Found.	Percentage.
mg.	*mg.*	*per cent*
0.1202	0.1036	86
0.1202	0.1050	88
0.698	0.609	87
0.698	0.605	87
0.698	0.610	87
1.396	1.215	87
1.396	1.215	87
2.792	2.405	86
2.792	2.395	86
6.01	5.05	84
6.98	5.90	84.5
12.02	10.25	85
24.04	20.90	87
39.53*	38.2	86

* Carried out by oxidizing for $2\frac{1}{2}$ hours.

was collected in a second Kjeldahl flask, and redistilled from sodium peroxide for 10 minutes into an Erlenmeyer flask. In both distillations a little water was present in the receiving flask, and the delivery tube dipped below the surface. Acetone was determined in the contents of the Erlenmeyer flask by the method described in an earlier paper (Hubbard, 1920). There was a blank amounting to 0.01 mg. of acetone.

Table I shows the results obtained by the method described. In these experiments pure solutions of calcium zinc β-hydroxybutyrate were weighed out, and aliquots were used for each

determination. The "amount present" is expressed as milligrams of acetone as calculated from the amount of this salt present, and the "amount found" represents the results of the titration corrected for the blank on the reagents. The recovery ranged from 84 to 88 per cent, and is approximately the same given by other methods in which sulfuric acid and potassium dichromate are used. The average of the figures is 86 per cent. Table I shows that the method as described is applicable for amounts of β-hydroxybutyric acid varying from 0.1 to 25 mg.

SUMMARY.

A method is described for the determination of β-hydroxybutyric acid when that compound is present in widely varying amounts. The oxidation requires only half an hour, and the final determination is by iodometric titration.

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DETERMINATION OF THE ACETONE BODIES IN URINE.*

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The determination of the acetone bodies in urine has been the subject of much investigation. Most of the methods for the determination of acetone described previously (Hubbard, 1920) and for the determination of β-hydroxybutyric acid described in the preceding note (Hubbard, 1921) have been used to determine the acetone bodies in urine, and, in many instances, were primarily developed for that purpose. Besides the references given in these two papers, other articles may be found in the bibliographies in papers by Shaffer (1908-09), Shaffer and Marriott (1913-14), Hurtley (1915-16), Van Slyke (1917), and Engfeldt (1920).

The present article contains the description of a method which has been found convenient for the determination of acetone from preformed acetone plus acetoacetic acid and from β-hydroxybutyric acid on the same sample of urine, even when they are present in very small amounts.

Preliminary Treatment.

In analyzing urine for the acetone bodies, particularly for β-hydroxybutyric acid, by any method, there are various interfering substances which must be removed. Shaffer (1908-09) removed these in two ways: first, by a preliminary precipitation; and second, by redistillation to remove compounds other than

* The work reported in this paper formed a part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Washington University, Saint Louis, in June, 1921.

acetone which react with alkaline iodine solutions. As a preliminary treatment before analysis he precipitated sugar and other interfering substances from urine with basic lead acetate and an excess of ammonium hydroxide. When this method was tried it was found that the filtrate often contained lead which was precipitated by subsequent treatment with sulfuric acid. Following a suggestion of Plimmer and Skelton (1914), sodium hydroxide was substituted for ammonium hydroxide, and it was found that glucose could be removed in concentrations up to about 5 per cent, and that lead could be completely precipitated at the same time, if the quantity of alkali was adjusted so that it was approximately equivalent to the lead present. During the study reported here a paper by Van Slyke (1917) appeared in which the use of copper sulfate followed by calcium hydroxide was recommended as a preliminary treatment before analysis. This treatment removed not only sugar, but other interfering compounds as well and was found necessary even when normal urines were analyzed by the technique described by him. An older method for removing sugar from urine was described by Salkowski (1879), in which copper sulfate and sodium hydroxide were used.

The following method was found to give almost complete removal of glucose and other easily oxidized compounds. 10 cc. of urine were measured into a 250 cc. graduate shaking cylinder, and diluted to 100 or 150 cc. 10 cc. of Goulard's extract[1] and 10 cc. of 20 per cent copper sulfate were added, followed by sodium hydroxide in not too great excess. (Usually 10 cc. of 2 N concentration were found to be the correct amount for the purpose.) This solution was diluted to 250 cc. and filtered after standing about half an hour. This combination of the lead and copper precipitation methods appeared, in some cases at least, to remove interfering (easily oxidizable) compounds more completely than did either procedure when used alone.

There is a relationship between the amount of glucose present in urine, and the amount of sodium hydroxide necessary to insure its removal by this technique. If 10 cc. of the Goulard's extract and 10 cc. of 20 per cent copper sulfate solution are added to 10 cc. of normal urine diluted with 100 cc. of distilled water, 5 cc. of twice normal sodium hydroxide will not precipitate all the lead

[1] $Pb_2O(CH_3 \cdot COO)_2$, 290 gm. to 1,000 gm. of solution, prepared according to U. S. P., 1916, ix, 249.

from the solution. Under the same conditions, 15 cc. will dissolve a part of the lead, while if 7 or 12 cc. are used no lead will be found after filtering. If 10 per cent of glucose is present in the urine, 15 cc. of 2 N sodium hydroxide will not cause the appearance of lead in the filtrate. 10 cc. of the alkali described above

TABLE I.

Precipitation of Urine.

Glucose.	NaOH	Found after precipitation.		
		Glucose.	Lead.	Copper.
per cent	*cc.*			
0.0	5		Trace.	0
0.0	7		0	0
0.0	12		0	0
0.0	15		Trace.	0
0.0	20		+	0
1.5	12	0	0	0
1.5	15	0	Trace.	0
2.5	7	Very faint trace.	0	0
5	7	Trace.	0	0
5	10	0	0	0
7.5	10	Trace.	0	0
10	10	"	0	0
10	12	Faint trace.	0	0
10	15	0	0	0
15	15	Faint trace.	0	0
20	12	+	0	0
20	15	Trace.	Trace.	Trace.
20	20	+	+	+

Glucose was added to normal urine to give the percentage listed. 10 cc. of each were measured into 250 cc. shaking cylinders and diluted to about 100 cc. 10 cc. of Goulard's reagent and 10 cc. of 20 per cent copper sulfate followed by different amounts of 2 N sodium hydroxide were added, and the solution was diluted to the mark and filtered at once. The filtrate was tested for glucose with Benedict's solution, for lead with an excess of sulfuric acid, and for copper with ammonia.

will remove glucose up to a concentration of about 5 per cent, while larger amounts—15 cc. of 2 N—must be used if the concentration is 10 per cent. Higher concentrations than this were not removed by the treatment, and urines containing more than 10 per cent of glucose must be correspondingly diluted before treatment. The facts discussed above are shown in Table I.

For purifying acetone distilled from acetone bodies present in urine Shaffer (1908-09) used different methods for the different fractions which he determined. He added sodium hydroxide to the distillate containing acetone from acetoacetic acid, and redistilled before titrating to remove volatile acids. To the fraction corresponding to β-hydroxybutyric acid he added hydrogen peroxide as well as alkali before redistilling to oxidize acetaldehyde and related compounds to the corresponding acids. Folin and Denis (1914) used sodium peroxide instead of hydrogen peroxide for this purpose. These methods are satisfactory for urines in which the acetone bodies are increased to any considerable extent, but for normal urines further treatment for the removal of interfering compounds was found to be necessary if the final determination was to be carried out with dilute alkaline iodine solutions. This further purification was accomplished by redistilling first from a solution of acid plus potassium permanganate, and then by distilling again from sodium peroxide.

The following directions describe the method used for the analysis of the filtrate from the copper and lead precipitation for acetone plus acetoacetic acid and for β-hydroxybutyric acid.

Determination of Acetone Plus Acetoacetic Acid.

Measure 150 cc. of the filtrate from urine precipitated as described into a 300 cc. Kjeldahl flask, add 10 cc. of sulfuric acid (1 part concentrated acid diluted with 1 part water), insert a two-holed rubber stopper, with a dropping funnel in one hole and a bent distilling tube in the other, connect with a condenser, and distil at such a rate that about 50 cc. of distillate come over in 10 minutes. Collect the distillate in a 500 cc. flask containing a little water with the end of the delivery tube below the surface, as it should be in all cases in which acetone solutions are distilled, and make the distillate up to a volume of about 150 cc. Add to the contents of this receiving flask 5 cc. of strong sulfuric acid (1 part concentrated acid plus 1 part water), 0.2 gm. of potassium permanganate, and distil, collecting the distillate in a second 500 cc. flask; continue distillation 10 minutes or more, obtaining a final volume of about 100 cc. and taking care that none of the permanganate solution boils over. Add to the contents of the

second distilling flask about 0.5 gm. of sodium peroxide, and distil 10 minutes into an Erlenmeyer flask containing a little water, collecting 50 to 100 cc. If care is not taken at the start, the solution will foam over. Cork stoppers should be used for this distillation. This technique insures maximum oxidation of interfering compounds and does not oxidize acetone (Hubbard, 1920). When more than mere traces of acetone are present, that is, when the urine gives a distinctly positive test with ferric chloride or with sodium nitroprusside and alkali, the purification by the successive redistillations is unnecessary; for these urines the single redistillation from alkali as recommended by Shaffer (1908-09) is most satisfactory (see Table II). This technique is to be preferred under these conditions, not only because it consumes less time and takes less apparatus, but also because it reduces chances of loss through the vaporization of acetone.

Determine acetone in the final distillate as follows (Hubbard, 1920): add 10 to 25 cc. of a solution of iodine in potassium iodide of such a strength that 1 cc. is equivalent to 1, 0.1, or 0.2 mg. of acetone (the concentration of the iodine to be used is indicated by preliminary qualitative tests on the urine); add 2 cc. of a concentrated solution of alkali (200 gm. of electrolytic sodium hydroxide dissolved in 300 cc. of distilled water), mix thoroughly, and allow to stand for 10 minutes or more; acidify with sulfuric acid, and titrate after about 5 minutes with sodium thiosulfate of a concentration equivalent to that of the iodine used; add a small amount of starch before the end-point is reached to serve as indicator. Control titrations of the thiosulfate against iodine treated successively with alkali and acid should be run daily, as the alkali uses up some of the iodine, and the strength of the latter reagent varies somewhat from day to day.

The difference between this control titration and the titration found after distillation measures the acetone present in the sample taken, equivalent to 6 cc. of urine. In cases where there is very little acetone present it is sometimes necessary to correct for a blank given by the reagents after distillation. The question of this correction is discussed later.

The stock iodine solution is prepared by weighing out 13.13 gm. of iodine, dissolving with the help of 25 gm. of potassium iodide, and diluting to 1 liter. Dilute solutions are prepared from this by diluting with 2.5 per

cent potassium iodide to the desired iodine concentration. These dilute solutions change their strength slowly.

The stock thiosulfate solution of an equivalent strength is made by dissolving 25.65 gm. of the pure salt in distilled water. This is standardized after 24 hours against an equivalent solution of potassium biiodate containing 3.362 gm. per liter, and protected from the action of carbon dioxide with soda lime; so protected, the solution will keep its strength unchanged for months. The dilute solutions are not as stable, and are prepared from this stock solution daily.

In Table II are given the values obtained for acetone from acetone plus acetoacetic acid as found after distilling a sample of normal urine, and of the filtrate from the copper and lead treatment of the same urine, from various oxidizing reagents. The

TABLE II.

Effect of Successive Distillation from Different Reagents on Urine Acetone.

Distilled successively from reagents given.	Straight urine in		Precipitated urine in	
	6 cc.	100 cc.	6 cc.	100 cc.
	mg.	*mg.*	*mg.*	*mg.*
H_2SO_4	0.76	12.7	0.6	10.0
H_2SO_4; NaOH	0.11	1.8	0.10	1.7
H_2SO_4; Na_2O_2	0.17	2.8	0.16	2.7
H_2SO_4; $H_2SO_4 + KMnO_4$	0.14	2.3	0.11	1.8
H_2SO_4; $H_2SO_4 + KMnO_4$; Na_2O_2	0.05	0.8	0.03	0.5
H_2SO_4; $H_2SO_4 + KMnO_4$; Na_2O_2	0.0462*	0.8*	0.0496*	0.8*

* Carried out on a different sample of urine and titrated with a weaker solution of thiosulfate. All results are expressed in terms of acetone.

repeated distillation is shown to be necessary when the amount of acetone is very small, but not necessary when it is increased as, in the latter case, the very small difference lies within the limits of experimental error. The table also shows agreement between figures on untreated urine and on filtrates from the precipitation with copper and lead when the distillate is purified by successive redistillations.

Determination of β-Hydroxybutyric Acid.

To determine the β-hydroxybutyric acid in the urine, treat the contents of the first distilling flask (urine filtrate plus sulfuric acid) by the technique described in the preceding note (Hubbard, 1921).

To the boiling solution add, through the dropping funnel, 20 cc. of the strong sulfuric acid (1 part concentrated acid plus 1 part water), 30 cc. of 0.1 to 0.2 per cent potassium dichromate, and continue the determination as described for solutions of pure β-hydroxybutyric acid. Redistil the acetone obtained as in the case of the first fraction from sulfuric acid plus potassium permanganate and from sodium peroxide, and carry out the determination on the final distillate in the manner described for the fraction from preformed acetone plus acetoacetic acid. This determina-

TABLE III.

Duplicates on Urines.

Redistillations.	Urine No.	Acetone + acetoacetic acid.		β-Hydroxybutyric acid.	
		Sample.	Per 100 cc.	Sample.	Per 100 cc.
		mg.	*mg.*	*mg.*	*mg.*
From Na_2O_2*............	1	0.067	0.7	0.180	1.8
	1	0.080	0.8	0.290	2.9
	1	0.077	0.8	0.200	2.0
	1	0.035	0.35	0.183	1.8
	1	0.045	0.45		
From H_2SO_4 + $KMnO_4$† and from Na_2O_2......	2	0.078	1.3	0.130	2.2
	2	0.084	1.4	0.130	2.2
	2	0.072	1.2	0.110	1.8
	2	0.096‡	1.6‡	0.110‡	1.8‡
	2	0.078‡	1.3‡		

* Samples of 10 cc. each used for these determinations.

† Samples of 6 cc. each used for these determinations.

‡ Glucose added to the urine to give a concentration of 5 per cent.

tion gives the acetone formed from the oxidation of the β-hydroxybutyric acid present. A correction of 15 per cent must be added to the result to make up for the incomplete recovery of acetone. If much acetone is present the redistillation from sulfuric acid plus potassium permanganate may be omitted.

Table III shows the agreement between duplicates obtained by this technique on both fractions of acetone from normal urine, as contrasted with the agreement when the redistillation from acid plus potassium permanganate was omitted.

Recovery of Substances Added to Urine.

Table IV shows the recovery of acetone, acetoacetic acid, and β-hydroxybutyric acid by this method. The acetone used was purified by repeated redistillation until the boiling point was constant. The acetoacetic acid was prepared by hydrolyzing acetoacetic ethyl ester with sodium hydroxide, aerating to remove acetone, and acidifying. The product was then analyzed by distilling from sulfuric acid, and redistilling from sulfuric acid plus potassium permanganate and from sodium peroxide to remove alcohol, and titrating the acetone formed by the usual Messinger method. The β-hydroxybutyric acid was prepared from calcium zinc β-hydroxybutyrate which was shown, by its optical activity, to be 99 per cent pure. A solution of this salt was acidified, set in plaster, and extracted with ether for about 10 hours. The ether was distilled off, and the β-hydroxybutyric acid taken up with water. The solution was boiled with a little bone-black, filtered, made up to 100 cc. with distilled water, and read in a polariscope. The reading in a 2.2 dm. tube was 0.865. This corresponds to a concentration of 1.630 gm. in 100 cc. (two determinations).

$$\frac{0.865}{2.2 \times 24.12} = 1.630$$

Two dilute solutions were prepared from this by diluting 1 to 10. A 5 cc. portion of each was analyzed by the technique described in the preceding note (Hubbard, 1921), and it was found that the acetone recovered was 85 per cent of the theoretical amount, the usual percentage recovered by this oxidation. The recovery of these substances when added to normal urine was satisfactory (Table IV).

Blanks on Reagents.

In all determinations in which distillation precedes the final analysis with dilute iodine solutions there is a blank. Its value is small when measured in terms of milligrams of acetone, but may amount to a high percentage of the total determinations when very small quantities of acetone are present. A large number of experiments were carried out to determine the source of this blank, and to find out conditions which should reduce it to

a minimum. Many of the precautions which were used to obtain very low blanks have been since found described by Widmark (1919) in his paper on the determination of acetone in blood.

TABLE IV.

Recovery of Substances Added to Urine.

Concentration prepared per 100 cc.	Taken.		Found.	Calculated per 10 cc.	Added per 10 cc.	Per cent.
	Filtrate.	Urine.				
			Acetone.			
mg.	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	
2.6	150	6	0.088	0.146	0.144	101
5.8	150	6	0.178	0.296	0.289	102
14.8	150	6	0.424	0.707	0.739	96
30.4	150	6	1.00	1.67	1.52	110
60.0	150	6	1.88	3.13	3.00	104
149.0	150	6	4.32	7.20	7.45	97
600	150	6	17.5	29.2	29.98	97.5
			Acetoacetic acid.			
2.6	150	6	0.0656	0.109	0.108	100
25.8	150	6	0.834	1.39	1.29	108
60.6	150	6	2.07	3.46	3.32	104
117	150	6	3.82	6.37	6.41	100
132	150	6	3.87	6.47	6.64	98
320	150	6	9.55	15.92	16.01	100
638	150	6	19.67	32.70	31.97	102.5
684	150	6	18.23	30.4	34.2	90
			β-Hydroxybutyric acid.			
4.5	150	6	0.230	0.382	0.454	85
9.1	150	6	0.470	0.785	0.909	86
18.2	150	6	0.975	1.62	1.82	89
45.45	150	6	2.27	3.90	4.545	84
90.9	150	6	4.69	7.81	9.09	86
181.8	150	6	9.54	15.9	18.2	87.5
454.5	150	6	22.24	37.15	45.45	82

All results and figures are given in terms of acetone.

One source of the acetone value found in blank determinations seems to be the presence of a very small amount of some impurity in the reagents, possibly in the lead subacetate used in precipi-

tating the urine, but this forms only a small percentage of the total value. The larger part of the blank is present when water alone is distilled successively from the different reagents, and seems to come largely from the last distillation from sodium peroxide. Cork stoppers must be used to connect the distilling flask with the condenser, unless, as suggested by Widmark, an all glass still is available. It is necessary to boil water through this still before each determination, and it was found best to boil a solution containing the same amount of sodium peroxide in the flasks before they were used for this final distillation. This procedure again resembles that recommended by Widmark. The still and flask used in the distillation with acid and potassium permanganate were similarly boiled out each day to remove

TABLE V.

Date.	Acetone + acetoacetic acid.	β-Hydroxybutyric acid.
1920	*mg.*	*mg.*
Sept. 11	0.0125	0.0185
" 11	0.0125	0.0165
" 11	0.0138	0.0131
" 12	0.0147	0.0190
" 12	0.0262	0.0189
" 12	0.0151	0.0135

All results are expressed in terms of the acetone equivalent of the blank. These blanks were obtained by redistilling the first distillate from H_2SO_4 + $KMnO_4$ and from Na_2O_2 successively.

grease which might yield, on oxidation, substances reacting with alkaline iodine solutions. Another source of error which should be avoided is the presence in the air of the laboratory of ammonia, formaldehyde, reducing gases, and other fumes which react with alkaline iodine solutions. In working with dilute reagents such as are used compounds of this nature may cause serious complications. If, however, the analyses are carried out on urines in which the acetone content is only slightly increased, these precautions may be omitted. In Table V a number of determinations of blanks on urine reagents treated as in the determination are given; these blanks show good agreement with each other. It is noticeable that the values of the blanks are so small that they are of importance only when there is very little acetone present.

In Table VI the results obtained by titrating the distillates finally obtained from normal urines are compared with results obtained by the use of the reagent described by Scott-Wilson (1911). Acetone gives a turbidity with this reagent and the reaction is a very delicate one. The turbidity obtained from the acetone was matched against that produced by a known amount of acetone freshly distilled into the reagent. In some cases the turbidities were matched in Nessler tubes; determinations could be made to an accuracy of about 0.005 mg. by this technique. In other cases the solutions were read in the nephelometer (Marriott, 1913–14), and when the amounts of acetone were comparatively large, the colorimeter as described by Folin and

TABLE VI.

Comparison of Urine Acetone by Iodine and by Scott-Wilson Reagent.

No.	Acetone + acetoacetic acid.				β-Hydroxybutyric acid.			
	Scott-Wilson reagent.		Iodine titration.		Scott-Wilson reagent.		Iodine titration.	
	6 cc.	Concentration per 100 cc.	6 cc.	Concentration per 100 cc.	6 cc.	Concentration per 100 cc.	6 cc.	Concentration per 100 cc.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.033	0.55	0.033	0.55	0.090	1.5	0.113	1.9
2	0.025	0.4	0.023	0.4	0.083	1.4	0.075	1.25
3	0.027	0.45	0.032	0.6	0.130	2.2	0.120	2.0

Three normal urines used. All results are expressed in terms of acetone.

Denis (1914) was used to measure the turbidity. Good agreement was found by the two methods, and it seems certain that the results must represent acetone. Whether the source of that acetone in the determinations of β-hydroxybutyric acid was actually that acid present in very small amounts in the normal urines analyzed is a question which the results do not establish.

Table VII contains results obtained by the use of the method described on a number of urines. The cases included range from normal subjects to diabetics showing a moderately advanced degree of acetonuria. A few facts are noticeable. First, the values found for the normals are very low, in the vicinity of about 2 mg. per 100 cc. of urine, for acetone from all acetone bodies. These results correspond with the lowest values included in the literature.

TABLE VII.

Urine Determinations.

Urine No.	Sex.	Date.	Preformed acetone + acetoacetic acid.		β-Hydroxybutyric acid.		Remarks.
			mg. per 100 cc.	*gm. per 24 hrs.*	*mg. per 100 cc.*	*gm. per 24 hrs.*	
1	Male.		1.3		2.4		Normal mixed.
2	"		0.5	0.007	2.5	0.030	" case 1.
3	"		0.7	0.009	1.6	0.021	" " 2.
4	Female.		0.4	0.001	1.4	0.010	" child.
5	Male.		1.6	0.003	2.3	0.009	" " .
6	"		0.8	0.002	1.6	0.003	" "
7	Female.	Mar. 13, 1921	1.9	0.016	1.2	0.010	Arthritic.
8	"	Feb. 17, 1921	0.2	0.004	0.5	0.009	" case 3.
9	"	Mar. 11, 1921	3.2	0.026	2.0	0.016	" " 4.
10	"	Nov. 10, 1919	1.1	0.006	6.1	0.040	Overweight.
11	"	Dec. 7, 1920	19.6	0.192	19.4	0.190	Diabetic.
12	Male.	Aug. 15, 1919	0.4		1.3		"
		" 19, 1919	2.1	0.024	1.5	0.017	
13	Male.	" 17, 1919	0.5	0.009	1.2	0.020	Diabetic.
		" 19, 1919	1.5	0.025	1.7	0.028	
14	Female.	" 17, 1919	11.2	0.182	15.0	0.244	Diabetic.
		" 18, 1919	9.2	0.156	17.8	0.302	
		" 19, 1919	11.2	0.063	22.9	0.128	
15	Female.	" 18, 1919	15.9	0.312	20.5	0.403	Diabetic.
		" 19, 1919	7.2	0.163	4.9	0.110	
		" 20, 1919	3.3	0.092	4.0	0.011	
16	Female.	" 18, 1919	1.6	0.021	4.3	0.055	Diabetic.
		" 19, 1919	1.3	0.017	3.3	0.043	
		" 20, 1919	2.5	0.024	5.3	0.052	
17	Male.	" 15, 1919	6.4	0.204	6.3	0.203	Diabetic.
		" 17, 1919	5.1		8.8		
		" 18, 1919			6.24	0.176	
		" 19, 1919	3.0	0.101	1.7	0.057	
18	Female.	" 15, 1919	0.4	0.007	2.1	0.041	Diabetic.
		" 17, 1919	1.3	0.058	3.4	0.058	
		" 18, 1919	4.4	0.085	4.3	0.079	
		" 19, 1919	1.7	0.032	2.5	0.048	

TABLE VII—*Concluded.*

Urine No.	Sex.	Date.	Preformed acetone + acetoacetic acid.		β-Hydroxybutyric acid.		Remarks.
			mg. per 100 cc.	*gm. per 24 hrs.*	*mg. per 100 cc.*	*gm. per 24 hrs.*	
19	Female.	Aug. 15, 1919	1.3	0.037	2.2	0.065	Diabetic.
		" 17, 1919	1.5	0.052	2.7	0.092	
		" 18, 1919	2.7	0.041	3.2	0.069	
		" 19, 1919	0.5	0.012	1.2	0.027	
20	Male.	Nov. 30, 1920	24.3	0.467	20.8	0.400	Diabetic case 5.
		Dec. 1, 1920	34.3	0.686	54.3	1.28	
		" 2, 1920	49.4	0.673	137	1.86	
		" 3, 1920	56.1	0.822	137	2.00	
		" 5, 1920	61.0	1.12	150	2.77	
		" 7, 1920	75.5	1.32	199	3.48	
		" 9, 1920	53.0	1.08	107	2.49	

All results are expressed in terms of acetone.

Second, there is, in most normal cases more acetone from β-hydroxybutyric acid than from acetone plus acetoacetic acid. In cases in which the excretion of the acetone bodies is slightly increased these two fractions are nearly equal, and in some of these cases there is an excess of the fraction from preformed acetone plus acetoacetic acid over that from β-hydroxybutyric acid. In the cases in which larger amounts of these substances were excreted the fraction from β-hydroxybutyric acid was again found to be the larger, as has been repeatedly found in cases of diabetes in which there was a marked degree of acetonuria. These facts militate against the theory formerly held that there is any definite ratio between the amounts of the acetone bodies formed in the body which can be studied from the proportions excreted (see also Hurtley, 1915–16). In the two following experiments it is shown that these same relationships occur in the same individual under conditions which lead to a gradual development of acetonuria.

In Table VIII[2] are recorded data obtained on a normal subject while he was living on a diet the fat content of which was increased.

[2] This experiment was carried out in the metabolic ward of Barnes Hospital, Saint Louis, through the courtesy of Dr. William H. Olmstead.

TABLE VIII.

Date	Diet.							Urine per 24 hours.						
	Protein.		Fat.		Carbohydrate.		Calories.	Volume.	Total N.	NH_4 N	Acetone + diacetic acid.		β-Hydroxybutyric acid.	
1917	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*cc.*	*gm.*	*gm.*	*mg. per 100 cc.*	*gm.*	*mg. per 100 cc.*	*gm.*
Mar. 12	96	15	201	69.5	100	15.5	2,573	935	12.5	0.748	0.7	0.006	2.2	0.020
" 13	96	15	201	69.5	100	15.5	2,573	1,100	17.0	0.748	4.4	0.048	3.8	0.042
" 14	96	15	201	69.5	100	15.5	2,573	905	16.6	0.776	7.2	0.065	3.5	0.032
" 15	68	10.1	251	82.4	50	7.5	2,711	1,095	17.8	0.787	34.2	0.374	18.8	0.206
" 16	68	10.1	251	82.4	50	7.5	2,711	815	14.6	0.815	58.0	0.473	69.0	0.562
" 17	68	10.1	251	82.4	50	7.5	2,711	1,180	12.5	0.914	68.0	0.802	112.0	1.42
" 18	72	11.7	175	63.7	150	24.6	2,448	980	13.4	0.964	28.0	0.274	35.9	0.352
" 19	72	11.7	175	63.7	150	24.6	2,448	1,040	10.3	0.849	10.3	0.107	5.8	0.061
" 20	72	11.7	175	63.7	150	24.6	2,448	860	9.75	0.670	3.3	0.026	3.4	0.029
" 21	72	11.7	175	63.7	150	24.6	2,448	810	10.6	0.667	2.8	0.023	3.4	0.026
" 22	72	11.7	175	63.7	150	24.6	2,448	610	9.94	0.490	1.4	0.024	4.0	0.024

Results of determinations of the acetone bodies are expressed in terms of acetone.

The subject of the experiment (the author), was a man 5 feet, $10\frac{1}{2}$ inches in height, weighing 165 pounds, who was doing light laboratory work at the time. The amounts of fat eaten were as follows: 200 gm. of fat during the first 3 days of the experiment; 250 gm. during a second period of the same duration; and 175 gm. during an after period of 5 days. The carbohydrate was also varied during the different periods as shown. There was a slight increase in the acetone excretion during the first period, a marked increase during the second period, and a return to practically normal values at the end of the experiment. Since the appearance of a paper by Shaffer (1921) on the relationship of glucose and fat to each other in diets which show acetonuria, the percentage of the total calories fed as fat, as protein, and as carbohydrate have been calculated, and are given in the table. It is noticeable that the first diet taken, which caused a slight increase in the excretion of acetone, was markedly higher in actual and available carbohydrate than the one which he has described as the border-line diet for acetonuria; that is, one containing the foods in the ratio of 10 per cent in the form of carbohydrate, and 80 per cent in the form of fat. The excretion of acetone on this diet was increased only very slightly, however, and can, perhaps, be properly attributed to variations in the mixtures of fat and carbohydrate burned at different times during 24 hours; with diets at or on the border-line of producing acetone it is evident that such variations may be important in causing slight increases in the excretion of acetone.

The relationship between the two fractions of acetone referred to above is shown in this experiment. At the start there was an excess of acetone from β-hydroxybutyric acid over that from acetone plus acetoacetic acid, a condition often, although not invariably, found in normal urine. During the first part of the experiment the fraction from acetone was in excess, but when the diet was markedly high in fat, and the total excretion of acetone consequently increased, the relationship of the substances was the same as that found at the start of the experiment; that is, there was an excess of acetone from β-hydroxybutyric acid similar to that usually described in diabetic urines. When the diet became more nearly normal, and the excretion of acetone began to decrease, there was again a period in which there was more acetone

from preformed acetone plus acetoacetic acid than there was from β-hydroxybutyric acid.

The total nitrogen (determined by the Kjeldahl method) shows a slight negative balance during the first part of the experiment, but this negative balance was not sufficiently pronounced to allow conclusions to be drawn from the data. The ammonia nitrogen excretion was increased, and roughly paralleled the increased acetone excretion. The ammonia was determined by the method of Folin and Macallam (1912).

Table IX, which contains results from another normal subject observed during a short fast, shows somewhat the same picture

TABLE IX.

Acetone Bodies Excreted during a Short Fast.

Day of fast	Before.*	First.	Second.	Third.	Day after.
Urine volume, *cc*	1,340	1,120	1,020	1,265	1,270
Acetone + acetoacetic acid, *mg. per 100 cc*	0.7	0.4	11.0	14.0	4.5
Acetone + acetoacetic acid, *gm. per 24 hrs*	0.009	0.0045	0.112	0.177	0.057
β-Hydroxybutyric acid, *mg. per 100 cc*	1.6	1.4	4.0	9.0	3.8
β-Hydroxybutyric acid, *gm. per 24 hrs*	0.021	0.016	0.041	0.114	0.043

* This sample of urine was obtained from the subject several days before the experiment was commenced.

All results are expressed in terms of acetone.

although here the experiment was not carried far enough to show the second crossing of the curves which is known to take place in cases of prolonged fasting. This case resembles, in the general course of the relative concentrations of the fractions, the cases reported by Folin and Denis (1915).

Many cases of diabetes studied during the development and clearing up of acetonuria have shown similar phenomena, and it can be found also in some of the results in the literature (Hurtley, 1915–16).

As it is generally accepted that acetoacetic acid is the immediate source of preformed acetone (Folin, 1907; Widmark, 1920) these two compounds can be considered together, and the acetone of

the breath added to give a total measure of the acetoacetic acid excreted. From the data presented, there would appear to be a marked increase in the acetoacetic acid before there is an increase in β-hydroxybutyric acid; if this fact, shown by a study of the excretion of the "acetone bodies," is a true measure of the relative formation of the two compounds in the body, it would seem to indicate that the substance which finally fails to be completely burned under the conditions studied is acetoacetic acid rather than β-hydroxybutyric acid. On the other hand, it is possible that the excretion of acetoacetic acid occurs at a lower concentration of the substance in the blood than does the excretion of β-hydroxybutyric acid. This is known to be the case for acetone itself as compared with acetoacetic acid (Widmark, 1920), and it is not unreasonable to assume that there may be a similar relationship between the "kidney thresholds" for the other two acetone bodies. In one case a sample of blood was obtained from a patient during the development of acetonuria when there was a higher concentration of acetone from acetone plus acetoacetic acid in the urine than there was from β-hydroxybutyric acid, and in the blood these two fractions were found to stand in the opposite relationship to each other. For the present, attention can only be called to the fact that there is such a difference in the excretion of the two fractions when the organism is producing only slightly increased amounts of the acetone bodies.

CONCLUSION.

A method has been described for the determination of the acetone bodies in normal urine, which gives a good percentage of recovery for substances added, and which is particularly applicable for the analysis of normal urines. It gives low values for normal urines, but not lower than some already included in the literature. Duplicates agree well, and determinations when carried out by two distinct methods of final analysis show satisfactory agreement. In addition two cases are presented in which the gradual development of acetonuria was brought about, and a brief discussion is given of the relationship between the different acetone bodies under such conditions.

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DETERMINATION OF THE ACETONE BODIES IN BLOOD.*

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Comparatively few methods are available for the determination of the acetone bodies in blood. Marriott (1913-14, *b*) has described a method by which the nephelometric technique described by him (1913-14, *a*) could be applied to the determination of these substances in blood. Scott-Wilson's reagent was used in this determination (Scott-Wilson, 1911). Marriott used this method in studying the relationships of the acetone bodies to each other (1914, *b*) and the level of acetone content of the blood in acidosis (1914, *a*).

Kennaway (1914) has described a method for determining acetone bodies in urine, using the Scott-Wilson reagent for the final determination. In a later paper Kennaway (1918) has applied this technique to the determination of acetone in the blood from diabetic patients, but was not able to carry through the method successfully on normal bloods.

Van Slyke and Fitz (1917, 1920) have described the application of Van Slyke's (1917, *b*) method to the determination of acetone bodies in blood. Short (1920) has recently described a modification of this method applicable to blood from operative cases in which ether is used.

Widmark (1919) has described a method for determining acetone from preformed acetone plus acetoacetic acid by the use of a

* The results reported in this paper formed a part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Washington University, Saint Louis, in June, 1921. A partial preliminary report of the method was given before the American Society of Biological Chemists, in December, 1920 (Hubbard and Wright, 1921).

modified Messinger titration method. This method, in common with those described by Ljungdahl (1917, 1919) and others, used a solution of biiodate as the source of the iodine. Widmark's method does not show the presence of acetone from preformed acetone plus acetoacetic acid in normal blood, and, in common with most of the other methods, is not applicable for the determination of β-hydroxybutyric acid in the same sample in which acetone is determined.

In extending the method for the determination of small amounts of acetone by the Messinger titration (Hubbard, 1920) to the determination of the acetone bodies in blood the only difficulty found was in choosing a satisfactory method for the precipitation of protein and other interfering compounds. Marriott (1913-14, b) used colloidal iron for removing protein before determining acetone with Scott-Wilson's reagent, and by modifying this procedure it was found possible to remove a large part of the reducing compounds with the protein, and so make the technique available for a final determination with alkaline iodine solutions instead of with the reagent used by him. The method used was based on experiments with lead and sodium hydroxide described in the preceding paper (Hubbard, 1921).

An amount of blood varying from 1 to 5 cc. was measured into 150 a ~~100~~ cc. shaking cylinder, and water was added to give a volume of between 40 and 50 cc. 50 cc. of colloidal iron were added, and the solution was thoroughly shaken. Next, 10 cc. of Goulard's extract were added, and the solution was shaken again. Finally, enough sodium hydroxide was added to precipitate the lead, the solution again thoroughly mixed, and allowed to stand for about 1 hour. It was then diluted to the mark and centrifuged in tubes covered with rubber caps to prevent loss of acetone. The supernatant liquid was then poured through filter paper, and an aliquot used for the analysis. The filtrate was clear, gave no precipitate of lead with sulfuric acid, or of protein with sulfosalicylic acid, and contained very little reducing substances when tested with alkaline picrate solutions even when the concentration of blood sugar was as high as 0.3 per cent.

The amount of sodium hydroxide that should be used must be determined by preliminary experiment for each batch of Goulard's reagent as the lead content of this reagent varies some-

what. The proper amount to use is the smallest amount of sodium hydroxide which will precipitate the lead from solution. The filtrate from the precipitation of lead with sodium hydroxide should react alkaline to litmus, but only faintly alkaline to phenolphthalein. 50 cc. when titrated with 0.01 N acid will require only about 2 cc. to render it neutral to phenolphthalein. 10 cc. of the reagent used in this work required 5.9 cc. of 2 N sodium hydroxide to give the required end-point. Electrolytic sodium hydroxide should be used in preparing this reagent.

An aliquot of the filtrate was used for the determination of the acetone bodies. If only 5 cc. of blood were available for the determination, the aliquot taken was usually 50 cc. When more blood was available two or three separate precipitations were carried out as described, and the filtrates combined. In all cases the solution was diluted to a final volume of 150 cc. for the determination. The accuracy of the determination is, of course, greatly increased when more blood is available. This analysis was carried out in the same way that was described for urine, except that it was found that a distillation of the distillate first obtained from an excess of sodium hydroxide before treatment with sulfuric acid plus potassium permanganate gave more consistent results. This treatment appeared to remove some volatile acid which, on oxidation with acid and potassium permanganate gave a compound which reacted with alkaline iodine solutions. Subsequent distillations from sulfuric acid plus potassium permanganate and from sodium peroxide were carried out as described in the preceding paper. For the final determination it is advisable, in most cases, to use 10 cc. of an iodine solution, 1 cc. of which is equivalent to 0.02 mg. of acetone, and titrate with a solution of sodium thiosulfate which is one-half as concentrated. In this case the correction to be added to the determination of acetone from β-hydroxybutyric acid is 17 instead of 15 per cent (see Table I).

Table I gives the recovery of the acetone bodies when added to blood. These compounds were prepared in the way already described in the paper on the determination of the acetone bodies in urine, and the percentage of recovery is practically the same as that found in the former study.

Table II gives the values found for determinations carried out on the reagents alone. These were carried out in the same

way that has already been described in the paper on urine, except for the extra preliminary distillation of both fractions mentioned above. There is good agreement among the duplicate determinations even when these are carried out on different days. It is

TABLE I.

Recovery of Substances Added to Blood.

Concentration prepared.	Taken.		Found.	Calculated per 5 cc.	Added per 5 cc.	Per cent.
	Filtrate.	Blood.				
Acetone.						
mg. per 100 cc.	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	
8.6	50	2½	0.233	0.446	0.432	106
31.0	50	2½	0.735	1.470	1.55	95
80.1	50	2½	1.93	3.8	4.05	95
160.2	50	2½	3.83	7.66	8.10	95
Acetoacetic acid.						
2.0	50	2½	0.0585	0.107	0.101	106
5.6	50	2½	0.143	0.286	0.285	100
11.3	50	2½	0.255	0.510	0.564	90.5
61.0	50	2½	1.54	3.08	3.05	101
119.4	50	2½	2.96	5.92	5.97	99
β-Hydroxybutyric acid.						
0.9	60	3	0.026	0.0434	0.0454	96
1.8	60	3	0.046	0.0767	0.0909	84
3.6	60	3	0.089	0.149	0.182	83
9.1	60	3	0.228	0.380	0.4545	84
18.2	60	3	0.433	0.723	0.909	80
18.2	50	2½	0.385	0.741	0.909	82
36.4	50	2½	0.741	1.48	1.82	82
44.4	50	2½	0.936	1.87	2.27	82.5
90.9	50	2½	1.83	3.65	4.545	81

All calculations are made in terms of acetone.

not necessary to run a blank on the reagents every time that a determination is carried out if the precautions described in the preceding paper are observed. In other words, the last flask must be freshly boiled out with sodium peroxide, a cork stopper

must be used in connecting that flask with the still, and, most important of all, the laboratory must be free from fumes.

Tables III and IV show that good agreement on duplicate samples of blood can be obtained by this technique.

TABLE II.

Blanks on Blood Reagents.

Date.	Filtrate.	Acetone + acetoacetic acid.		β-Hydroxybutyric acid.	
		Found.	Per 50 cc. filtrate.	Found.	Per 50 cc. filtrate.
1920	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*
Nov. 13	150	0.0147	0.0049	0.0262	0.0087
" 13	100	0.0112	0.0056	0.0182	0.0091
" 13	50	0.0082	0.0082	0.0107	0.0107
" 13	50	0.0082	0.0082	0.0097	0.0097
" 13	50	0.0092	0.0092	0.0137	0.0137
" 13	50	0.0067	0.0067	0.0072	0.0072
" 17	150	0.0205	0.0068	0.0165	0.0055
" 17	100	0.0155	0.0077	0.0235	0.0117
" 17	50	0.0065	0.0065	0.0135	0.0135
" 17	50	0.0065	0.0065	0.0110	0.0110
" 17	50	0.0125	0.0125	0.0095	0.0095
" 17	50	0.0070	0.0070	0.0225	0.0225*

All results are expressed in terms of the acetone equivalent of the blank. When determinations were carried out in the routine manner on 50 cc. of filtrate (equivalent to 2.5 cc. of blood) the difference between the most widely varying blanks from preformed acetone plus acetoacetic acid is equivalent to a difference of 0.1 mg. in 100 cc. of blood; under the same conditions the maximum variation for the blank from the β-hydroxybutyric acid fraction is 0.6 mg. in 100 cc.; if the starred (*) value is omitted, this difference of 0.1 and 0.2 mg. is based on eight and on seven separate determinations, respectively.

These blanks were obtained by redistilling the first distillate from NaOH, H_2SO_4 + $KMnO_4$, and Na_2O_2, successively.

It was thought that possibly small amounts of acetoacetic acid might be precipitated by the preliminary treatment of blood. It was possible that substances in blood might be lost in this way, although, when larger amouuts were added, such added amounts might be recovered. To test this possibility a series of bloods ranging from an acetone content of 0.05 mg. per 100 cc. to a content of 2.5 mg. per 100 cc. was tested by the following technique:

TABLE III.

Analysis of β-Hydroxybutyric Acid in Samples of Defibrinated Beef Blood.

Titration of iodine solution.	Titration of distillate.	Difference.	Acetone.	Blank equivalent.	Acetone corrected for blank.
cc. 0.01035 N thiosulfate	*cc. thiosulfate*	*cc. thiosulfate*	*mg.*	*mg. acetone*	*mg.*
9.80	8.90	0.90	0.090		0.050
9.80	8.95	0.85	0.085	0.04	0.045
24.60	23.75	0.85	0.085		0.045
24.60	23.65	0.95	0.095		0.055

TABLE IV.

β-Hydroxybutyric Acid in 100 Cc. Defibrinated Beef Blood.

Sample taken for analysis.	Acetone found.	Corrected for 80 per cent recovery.
cc.	*mg.*	
5	3.6	4.5
5	3.6	4.5
5	3.6	4.5
5	3.7	4.6
1	3.6	4.5

The sample of blood gave 0.5 mg. of acetone from preformed acetone and acetoacetic acid.

TABLE V.

Effect of Precipitation on the Determination of Acetone in Blood.

Blood taken.	Acetone found.		Taken.		Acetone found.	
			Filtrate.	Blood.		
cc.	*mg.*	*mg. per 100 cc.*	*cc.*	*cc.*	*mg.*	*mg. per 100 cc.*
5	0.0605	1.2	50	2½	0.0240	1.0
5	0.135	2.7	50	2½	0.0695	2.7
5	0.0953	1.9	50	2½	0.0417	1.7
5	0.0133	0.3	50	2½	0.00944	0.4
5	0.142	2.8	100	5	0.138	2.8
5	0.0020	0.04	100	5	0.0025	0.05
5	0.0565	1.1	100	5	0.0497	1.0

Results are acetone from preformed acetone plus acetoacetic acid expressed in terms of acetone.

Each pair of determinations was carried out in different bloods. All were purified by redistillation as described.

5 cc. of blood were measured into an 800 cc. Kjeldahl flask, about 200 cc. of water and 10 cc. of sulfuric acid (1 part of the concentrated acid diluted with 1 part of water) added, and the mixture was distilled for 20 minutes. The distillate was then purified by successive redistillation from sodium hydroxide, sulfuric acid plus potassium permanganate, and sodium peroxide. Duplicate samples of the same bloods were analyzed by the technique described in this paper; that is, they were precipitated and aliquots of the filtrate were distilled and redistilled as described. Table V contains the results obtained, and shows that there is good agreement between the values from the treated and untreated blood. In view of the recovery of acetone and of acetoacetic acid when added to blood before precipitation, and of the agreement shown

TABLE VI.

Comparison of Blood Acetone by Iodine and by Scott-Wilson Reagent.

No.	Taken.		Scott-Wilson reagent.		Iodine titration.	
	Filtrate.	Blood.				
	cc.	*cc.*	*mg.*	*mg. per 100 cc.*	*mg.*	*mg. per 100 cc.*
1	50	$2\frac{1}{2}$	0.004	0.2	0.005	0.2
2	50	$2\frac{1}{2}$	0.008	0.3	0.0094	0.3

Acetone from preformed acetone plus acetoacetic acid determined on two normal bloods.

between results on blood and the filtrate from the precipitation, it is certain that these compounds are not removed even in small amounts by the precipitation with colloidal iron, basic lead acetate, and sodium hydroxide.

The acetone plus acetoacetic acid from two bloods was determined by both iodine titration and precipitation with Scott-Wilson's reagent. The results are given in Table VI, and show good agreement between the values obtained. The analysis with Scott-Wilson's reagent was carried out as described in the preceding paper.

Table VII presents the results obtained on a series of bloods from different types of cases. It contains, in addition to the values of the acetone bodies, the values for blood sugar as determined by the method of Benedict (1918) and of carbon dioxide-

TABLE VII.

Blood Determinations.

Blood No.	Sex.	Date.	Acetone + aceto-acetic acid expressed as acetone.	β-Hydroxy-butyric acid.	Sugar.	Alkali reserve.	Remarks.
			mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	*vol. per cent*	
1	Male.	Nov. 19, 1919	0.3	0.8			Normal.
1	"	Dec. 8, 1919	0.1	0.1	0.098	65.3	
1	"	Jan. ?, 1920	0.1	0.4			
1	"	Nov. 11, 1920		0.4	0.098	74.0	
2	"	Dec. 17, 1919	0.1	0.3	0.121	71.0	Normal.
2	"	Nov. 18, 1920	0.7	0.4	0.135	68.3	
3	"	Dec. 14, 1919	0.3	0.4	0.125	76.8	Normal.
4	"	Nov. 27, 1920	0.3	0.4	0.102	78.0	"
5	"	Dec. 3, 1920	0.2	0.3	0.125	61.7	"
6	Female.	Nov. 5, 1919	0.8		0.118	61.5	"
7	"	Dec. 10, 1919	0.6	0.7	0.111	56.8	Obese.
8	"	Feb. 17, 1921	0.9	0.02	0.139	58.7	Arthritic.
8*	"	" 20, 1921	1.8	2.9	0.153	51.0	
9	"	Nov. 24, 1920	0.6	0.4	0.128	50.4	Nephritic.
10	Male.	Dec. 18, 1920	1.4	1.6	0.167	46.2	"
11	"	Nov. 6, 1920	1.1	0.8		62.4	Gastrointestinal.
12	Female.	Dec. 11, 1919	0.7	2.5	0.098	56.0	Thyroid.
13	"	" 10, 1919	0.03	0.2	0.102		"
14	"	Nov. 29, 1919	0.1	0.6	0.109	66.0	"
15	"	Dec. 7, 1919	0.2	0.3	0.125	78.7	"
16	"	Nov. 24, 1920	0.8	0.9	0.222	72.1	Diabetic.
17	"	Dec. 17, 1919	0.8	0.6	0.190	50.0	"
17	"	Jan. 9, 1920	1.2	1.4	0.125	67.2	
18	Male.	Dec. 4, 1920	6.5	8.9	0.222	42.8	Diabetic.
18	"	" 18, 1920	2.7	5.2	0.154		
19	Female.	" 10, 1920	1.0	1.0	0.250	57.9	Diabetic.
19	"	" 18, 1920	1.2	3.1	0.190		
20	"	" 7, 1920	1.0	2.4	0.161	57.6	Diabetic.
21	"	Nov. 11, 1919	0.0	0.4	0.266		"
22	Male.	" 11, 1919	0.9	0.0	0.144		"
23	"	Jan. 10, 1920	2.8	3.7	0.185		"
24	Female.	Aug. 18, 1919	0.7	2.0	0.128	57.0	"
25	"	" 18, 1919	1.5	1.5	0.092		"
26	"	Dec. 5, 1920	0.8	0.7	0.156	48.5	"

* After diet markedly high in fat fed for 4 days.

Results for acetone, acetoacetic acid, and β-hydroxybutyric acid are expressed as acetone.

Alkali reserve is measured as the CO_2-combining capacity of the plasma.

combining power of the plasma as determined by the method of Van Slyke and Cullen (Van Slyke, 1917, *a*; Van Slyke and Cullen, 1917). The bloods analyzed were kept from clotting with potassium oxalate, and were analyzed on the same day that they were taken. In most cases the bloods were drawn before breakfast. Inspection of the table shows that the values for normal bloods range from 0.1 to about 1.0 for acetone from all three acetone bodies. Results on cases of diabetes sometimes fall into the same range, and are sometimes much higher. Neither in normal nor in pathological specimens is there any relationship between the amount of acetone from acetone plus acetoacetic acid and that from β-hydroxybutyric acid except in the case of diabetic bloods in which both values are high; in them the acetone from β-hydroxybutyric acid is in excess.

CONCLUSION.

A method for the determination of the acetone bodies in blood has been described which gives a high and constant percentage of recovery for added acetone bodies, and which gives good agreement between duplicate determinations. Agreement is also found between the values of the acetone as determined by two different methods—a fact which renders it probable that the substance so determined is acetone. The results obtained by this method on blood from normal subjects are low. The accuracy of the determination is about 0.1 mg. per 100 cc. of blood.

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BLOOD ACETONE BODIES AFTER THE INJECTION OF SMALL AMOUNTS OF ADRENALIN CHLORIDE.*

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In an earlier paper (Hubbard, 1921) results were reported on the study of normal subjects under conditions which caused a slightly increased excretion of the acetone bodies. The results showed that under such conditions there was an amount of aceto-acetic acid excreted during the development of acetonuria which was in excess of the β-hydroxybutyric acid simultaneously excreted. Conclusions from these findings were uncertain, as the results were complicated by differences in the kidney thresholds of the different compounds, and these prevented certain interpretation of conditions within the organism. For this reason it seemed desirable to investigate conditions other than dietary changes which might give rise to increased production of the acetone bodies, and in which the increase and return to normal values would take place within a comparatively short period of time. The effect of the injection of adrenalin chloride was selected for study.

Peters and Geyelin (1917) have reported experiments which showed that after the injection of adrenalin chloride solution there were changes in the carbon dioxide-combining capacity of the plasma as well as in the blood sugar content and blood pressure. These experiments indicate that there are extensive changes in the chemistry of the blood brought about by the presence of large amounts of this substance, and it was thought that changes might be found in the acetone bodies. Eiselt (1910) has reported an increase in urine acetone as the result of the injection of adrenalin into a patient suffering from Addison's disease.

* A preliminary report of the work described below was given before the American Society of Biological Chemists, in December, 1920 (Hubbard and Wright, 1921).

A series of seven experiments was run on normal men. Each subject was fed a standard simple breakfast, and an hour afterwards received an injection of 0.5 or 1 cc. solution of adrenalin chloride in a one to one thousand dilution. A sample of blood was taken before the injection was given, and other samples were taken at various intervals after the injection. Each sample was analyzed for acetone from preformed acetone plus acetoacetic acid and for acetone from β-hydroxybutyric acid by the technique described in the preceding paper (Hubbard, 1921), for sugar by the technique described by Benedict (1918), and for the carbon dioxide-combining capacity of the plasma by the method of Van Slyke and Cullen (Van Slyke, 1917; and Van Slyke and Cullen, 1917). Changes in the systolic and diastolic blood pressure and the pulse rate were also recorded. These last showed no anomalies, except the expected variations with the larger dose of adrenalin chloride, and a detailed description of them has accordingly been omitted from this paper.

The results obtained from the chemical analysis of the various samples of blood on the different patients are listed in Table I. Three of the experiments show a distinct rise of the acetone bodies, with a subsequent return to the values found preceding the injection of the adrenalin. In two of these cases both of the fractions show the increase. The experiments which showed the most marked variations are experiments in which the subject received a dose of 1 cc. of the drug. There was one experiment in which a dose of 1 cc. was given, and in which there was no change in the acetone bodies. The subject of this experiment showed a high blood sugar value in the sample taken before the injection and perhaps should not be classed as normal but there is no other reason for making such an assumption. There is no constant relationship in the degree of response of the different acetone bodies, and the results in the cases where there is a rise noted do not seem to bear any relationship to the changes in blood sugar nor in the carbon dioxide-combining power of the plasma. The magnitude of the rise observed in some cases and the subsequent return to normal values indicate that the changes are real changes in the substances present in the blood, induced by the adrenalin chloride administered.

TABLE I.

Case No.	Sex.	Date.	Dose $\frac{1}{1000}$	Time.	Acetone plus aceto-acetic acid per 100 cc.	β-Hydroxybutyric acid per 100 cc.	Sugar.	Plasma bicarbonate CO_2.
			cc.	*hrs.*	*mg.*	*mg.*	*per cent*	*vol. per cent*
1	Male.	Dec. 8, 1919	½	Before.	0.1	0.1	0.098	65.3
				½	0.3	0.3	0.156	
				1½	0.05	0.2	0.160	72.4
				2½			0.100	71.0
1	"	Nov. 7, 1920	1	Before.		0.25	0.098	74.0
				½	0.6	1.1	0.167	
				1	1.3	1.7	0.225	53.8
				2	0.5	0.2	0.136	51.0
2	"	Dec. 17, 1919	½	Before.	0.1	0.3	0.121	71.0
				½	0.1	0.3	0.133	71.0
				1½	0.25	0.4	0.222	69.1
				2½	0.1	0.4	0.160	71.0
2	"	Nov. 18, 1920	1	Before.			0.135	68.3
				½		0.3	0.157	59.8
				1	0.2	0.0	0.215	56.6
				2	0.2	0.2	0.194	55.1
3	"	" 14, 1919	½	Before.	0.3	0.4	0.125	76.8
				½	0.5	1.1	0.154	76.8
				1½	0.6	0.5	0.128	67.3
				2½	0.25	0.35	0.122	76.8
5	"	Dec. 3, 1920	½	Before.	0.2	0.3	0.125	61.7
				½	0.3	0.3	0.154	62.6
				1	0.6	0.1	0.200	62.1
				2	0.3	0.3	0.122	59.8
4	"	Nov. 27, 1920	1	Before.	0.3	0.4	0.102	65.3
				½	0.8	0.5	0.236	59.5
				1	0.8	0.9	0.266	55.7
				2	0.3	0.3	0.166	61.4

Results for all three acetone bodies are expressed as acetone.

Alkaline reserve is measured as the CO_2-combining capacity of the plasma.

The most satisfactory explanation for the results reported is in the probable local restrictions of the blood supply induced by the drug, which lead to a local production—or failure of combustion—of the acetone bodies. Such production could occur under these conditions in spite of the increased glucose content of the blood.

The production of these acetone bodies certainly cannot be looked upon as responsible in any degree for the marked lowering of the alkaline reserve observed.

The experiments do not afford any information concerning the question of the order of the production of the acetone bodies in the organism.

Our thanks are due to the members of the Staff of The Clifton Springs Sanitarium who served as subjects for these experiments, and particularly to the late Dr. Malcolm S. Woodbury, superintendent of the Sanitarium, for the continued encouragement which he extended to us during our experiments.

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SOME NUTRITIVE PROPERTIES OF NUTS.

II. THE PECAN NUT AS A SOURCE OF ADEQUATE PROTEIN.*

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(Received for publication, October 13, 1921.)

It has been recently observed that young rats would grow at a normal rate and attain adult size on diets in which the essential source of the protein of the ration was derived from various nuts.[1] From these results it was concluded that the proteins of these nuts furnished suitable amounts of those amino-acids necessary for growth and that they can be regarded as "complete" from the point of view of nutrition.

With the exception of the pecan nut successful feeding trials resulted with all the nuts investigated, a list that included many of our important protein-rich nuts. The rations containing the pecan nut as the source of protein were complete in every other known dietary essential. Two causes may account for the failure of rats to grow at a normal rate on such diets. The proteins of this nut may yield insufficient amounts of those amino-acids that determine the nutritive value of a protein; or the pecan nut may contain some substance which renders rations of which it is an important component distasteful or injurious to rats.

At the time of these observations there were on record neither detailed studies of the type of protein existing in the pecan nut nor studies of their chemical make-up. In view of the importance that this nut is assuming as a food crop in the United States,[2]

* The results reported in this paper were presented before the Pacific Coast Division of the Society of Experimental Biology and Medicine at the meeting of October 15, 1921.

[1] Cajori, F. A., *J. Biol. Chem.*, 1920, xliii, 583.

[2] In 1909 the production of pecan nuts in the United States was 9,890,769 pounds. In 1919, 45,619,000 pounds, a gain in 10 years of 400 per cent. These figures were taken from the 1910 United States Census Reports and the United States Bureau of Crop Estimation report, 1919.

it seemed desirable to study the chemical character of the proteins of the pecan nut, and with such a basis for evaluation of their nutritive properties, to repeat feeding experiments on animals.

Chemical Experiments.

Methods for the isolation and purification of plant proteins have been developed by Osborne[3] and more recently by Johns and his coworkers[4] in their investigations of seed proteins. We have followed these methods in our work on the proteins of the pecan nut. The Van Slyke method[5] was used for protein analysis.

Preliminary Experiments.

Several kilos of shelled pecan nuts (1.5 per cent nitrogen) were passed through a meat grinder and the finely divided mass put in a laboratory press. This process, followed by repeated extraction with petroleum ether, removed all but traces of oil. The oil-free residue was ground in a mortar; the resulting product was a fine gray powder containing 4.3 per cent nitrogen.

Small samples of the pecan meal were extracted over night with sodium chloride solutions of different strengths, and aliquots of the clear filtrate analyzed for nitrogen. Table I shows the amount of protein extracted from 2.5 gm. samples of the meal.

Fractional Precipitation with Ammonium Sulfate.—Solid, finely powdered ammonium sulfate was added to 10 cc. of a 9 per cent sodium chloride extract of pecan meal. The salt was added in amounts calculated to gradually saturate the solution, each addition increasing the degree of saturation by 0.5 per cent. After each addition of ammonium sulfate the solution was shaken and the salt completely dissolved before the next quota of salt was added. The solution was examined for precipitated protein at the various stages of saturation.

When the solution was 0.2 saturated a slight cloud appeared. At 0.4 saturation the solution became turbid and at 0.45 saturation a flocculent precipitate formed which was removed by filtration. Further addition of ammonium sulfate to the filtrate caused no change until 0.8 saturation was reached when the solution again became slightly turbid. This turbidity persisted, unchanged, when the solution was completely saturated. Examination of the saturated solution after it had stood over night showed that a small flocculent precipitate had formed.

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[3] Osborne, T. B., The vegetable proteins, London, New York, Bombay, and Calcutta, 1909.

[4] Johns, C. O., and Waterman, H. C., *J. Biol. Chem.*, 1920, xlii, 59. Johns, C. O., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1920–21, xlv, 57.

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15; 1915, xxii, 281.

To 5 cc. of the 9 per cent sodium chloride extract, 1.86 gm. of ammonium sulfate were added so as to make the solution 0.5 saturated. A heavy precipitate formed. After 2 minutes this was filtered and 0.74 gm. of ammonium sulfate was added, making the solution 0.7 saturated. A very slight turbidity was noticed. To completely saturate the solution 1.16 gm. of the salt were added. A marked turbidity resulted.

Temperature of Coagulation.—5 cc. of the 9 per cent sodium chloride solution were acidified with a drop of very dilute acetic acid. A thermometer was inserted in the test-tube containing the acidified solution and the tube heated in a double jacketed water bath. The temperature was so regulated that there was a rise of not over 1° in 2 minutes.

At 55°C. a slight turbidity was noted but no flocculent coagulum appeared until the temperature of 70°C. was reached. The solution was kept at 71°C. for 1 hour. After cooling, the precipitate was removed by filtration and the clear filtrate again heated. At 70°C. the solution became slightly cloudy, and at 79–82°C. a precipitate began to form. A definite coagulum formed at 86°C. and the solution was kept at this temperature for 1 hour. The heavy coagulum present at the end of this time was removed by filtering, and the heating continued. At 90°C. a slight cloudiness appeared.

TABLE I.

Extraction of Pecan Proteins with Sodium Chloride Solutions.

NaCl	N extracted.	Protein extracted (N × 6.25).
per cent	*mg.*	*per cent*
0.1	12.4	3.1
1.0	12.7	3.1
3.0	26.4	6.9
5.0	35.1	8.1
7.0	45.8	10.6
9.0	50.9	11.9
11.0	58.0	14.4

Long heating at 97–100°C. caused a slight coagulation to form.

The preliminary experiments indicate that pecan meal contains, as its principal protein, a globulin, salted out by 0.5 saturation with ammonium sulfate and coagulated at 79–86°C. There is evidence of a trace of an albumin, which starts to coagulate at 55–60°C. and is precipitated by ammonium sulfate only at a point of complete saturation. The fact that at the temperature of boiling water a slight coagulum forms, in addition to that caused by lower temperatures, is evidence, perhaps, of a second globulin, present in very small amounts.

Preparation of a Pecan Globulin.

Several hundred grams of pecan meal were extracted with seven times their weight of 10 per cent sodium chloride solution. The undissolved residue was separated from the solution by squeezing the mass through cheese-cloth. A solution free from suspended particles was obtained by filtering it several times through thick layers of paper pulp on a Buchner filter. This slightly opalescent solution was made 0.5 saturated with ammonium sulfate by the addition of the calculated quantity of the salt and allowed to stand over night. As much as possible of the clear supernatant solution was syphoned off from the precipitated globulin before it was transferred to a filter. The protein was removed from the filter paper and redissolved with distilled water and a little 10 per cent sodium chloride. It was again filtered clear and then dialyzed in parchment paper bags against running water for 120 hours. After that time it gave no more marked a test for sulfates and chlorides than did the tap water against which it was dialyzed. The contents of the bag were transferred to a Buchner filter and washed with distilled water and alcohol. The globulin was dehydrated by suspending it in absolute alcohol over night. It was filtered on a hardened filter paper, washed with anhydrous alcohol and ether, dried in a Freas oven at 110°C., and finally placed in a vacuum desiccator over concentrated sulfuric acid.

The pecan globulin, prepared in this way, was a light gray powder, with no evidence of crystalline structure, containing 15.76 per cent nitrogen and 0.83 per cent sulfur, calculated on a moisture- and ash-free basis. It gave the usual protein tests including a strongly positive test for tryptophane with the Hopkins-Cole reagent. With α-naphthol a very faint color developed due undoubtedly to slight contamination of the preparation with traces of filter paper.

The distribution of the nitrogen in this globulin as determined by the Van Slyke method, after complete hydrolysis with 20 per cent hydrochloric acid, is shown in Table II.

In general this analysis agrees fairly well with the recently published results of Dowell and Menaul.[6] These authors determined the nitrogen distribution of the mixed proteins extracted from pecan meal by barium hydroxide and sodium hydroxide. The fact that our analyses are similar would indicate that the globulin constitutes the large part of the proteins of the pecan nut. It may be noted that there is no evidence either in our

[6] Dowell, C. T., and Menaul, P., *J. Biol. Chem.*, 1921, xlvi, 437.

results or in those of Dowell and Menaul of the unusually high content of histidine or a low arginine content in pecan proteins, reported by Nollau,[7] in an analysis published some years ago.

In considering the nutritive value of the pecan nut, the large amounts of basic amino-acids yielded by the globulin are significant.

TABLE II.

Distribution of Nitrogen in Pecan Globulin.

After hydrolyzing 2.1727 gm. of the protein, the solution contained 341.9 mg. of N.

	mg.	*per cent*
Amide N	33.5	9.8
Humin N	12.3	3.6
Arginine N	78.3	22.9
Histidine N	12.7	3.7
Cystine N	2.7	0.8
Lysine N	21.2	6.2
Monoamino N	176.8	51.7
Non-amino N	2.7	0.8
Total	340.2	99.5

Feeding Experiments.

There is every indication from a chemical examination of the globulin of the pecan nut that this nut is of high biological value as a source of protein. It seems highly improbable that the failure of rats to grow, when the pecan nut furnishes the protein of their ration, can be attributed to an amino-acid deficiency of the diet.

The shelled pecan is bitter and astringent because there is a large quantity of tannin in the outside cuticle. In a recent study, Friedemann[8] has shown that tannins constitute 2.57 per cent of the total carbohydrates of the nut, and are present in sufficient amounts to give a tannin content of 0.33 per cent to the whole nut. The astringency that this amount of tannin gives to diets made up in large parts of pecan nuts or pecan press-cake may render such rations unsuitable for rats, and we have endeavored to remove as much tannin as possible from the nuts before incorporating them in our diets.

[7] Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.

[8] Friedemann, W. G., *J. Am. Chem. Soc.*, 1920, xlii, 2286.

Such a color test as the ferric chloride test shows that tannins are present exclusively in the outer layers of the pecan kernel, and removal of this layer should render the nut tannin-free. The integument of smooth nuts, such as the almond, can be removed by blanching with hot water. But in the case of the pecan nut this procedure fails to detach the membranous skin from the numerous crevices of the wrinkled surface of this nut. An endeavor was made to remove the outer layer by laboriously scraping each kernel with a knife blade. Again the irregular surface of the nut made it difficult to remove all the parts containing tannin. Leaching pecan meal with water did not prove successful as a means of removing tannin, and the possible loss of proteins during the leaching made this process undesirable.

With many fruits astringency has been successfully combated by the use of solutions of lye, and we have found that exposure of pecan nuts to hot solutions of sodium hydroxide would loosen the integument completely, and remove the layer containing tannins. Our procedure was as follows:

Pecan halves were added to a boiling 1 per cent solution of sodium hydroxide and the boiling solution was stirred vigorously for a minute. The mass was quickly transferred to a brass screen whose mesh was large enough to permit the alkaline solution to pass through readily but which would retain the pecan halves. Without delay the nuts were washed several times with boiling water, once with 1 per cent hydrochloric acid and again with hot water. In this way the sodium hydroxide was completely removed. When this process was carried to completion rapidly the solvent action of the alkali, and consequent loss of protein, was restricted to the outer layer of the nut and a white non-astringent product was obtained.

The nuts, after the lye treatment, were dried, passed through a meat grinder, and subjected to pressure to remove enough oil so as to obtain a press-cake of sufficiently high protein content to be incorporated in a diet at an 18 per cent level. Young rats were fed on rations containing this pecan press-cake, butter fat, and inorganic salts. In addition to this diet 1 gm. of pecan nut was fed daily to each rat to insure an adequate supply of water-soluble vitamine. Cajori[1] has shown that this dietary essential is furnished in sufficient amounts for normal nutrition in rats by that amount of pecan nut.[9]

[9] We are indebted to Mr. C. J. Moore of San Antonio, Texas, who kindly supplied us with some of the pecan nuts used in these feeding experiments.

As will be seen in Chart 1, the animals on this diet grew at a normal rate, indicating that the proteins of the pecan nut furnish adequate quantities of those nitrogenous complexes essential for growth. The weekly food intake records are given in Table III.

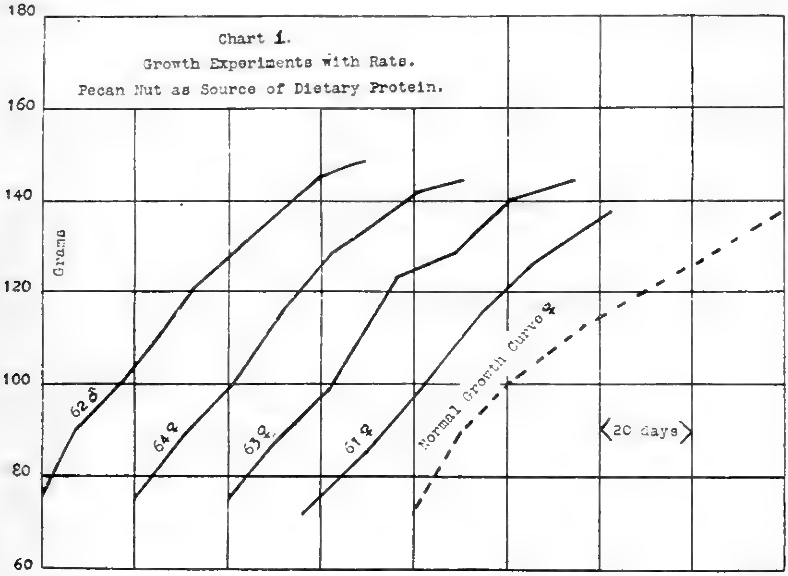

Chart 1. Growth of young rats on diets in which the pecan nut furnished the sole source of protein in the diet. The pecan nut was incorporated in the diet in the form of a press-cake after the tannin had been removed from the surface of the nut by treatment with hot sodium hydroxide.

The composition of the pecan nut diet was as follows:

	per cent
Pecan press-cake, 3.2 per cent N	90
Salt mixture*	3
Butter	5
Lard	2
Pecan nuts	1 gm. daily

* The salt mixture used is that described by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 317.

The failure of normal growth on diets containing pecan nuts from which the tannins have not been removed, is shown in Chart 2. In all respects except tannin content, these animals received the same diet as those whose growth is pictured in Chart 1.

TABLE III.

Weekly Consumption of Pecan Nut Diet.

Week.	Rat 61.	Rat 62.	Rat 63.	Rat 64.
	gm.	*gm.*	*gm.*	*gm.*
1	26	35	38	34
2	31	34	31	34
3	36	40	37	39
4	39	42	43	42
5	43	44	44	43
6	49	44	42	43
7	39	47	39	44
8	44	46	40	36
9	41	42	43	39
10	43	47	45	49
11	41		42	

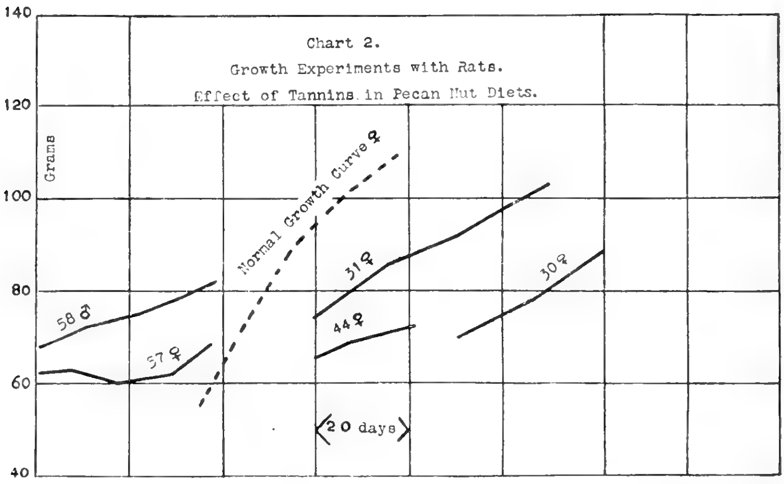

CHART 2. Failure of normal growth of young rats on diets in which the pecan nut furnished the sole source of protein in the diet. The tannin in the surface of the nut had not been removed completely before the nut was incorporated in the diet.

Rats 30 and 31 received a ration containing pecan press-cake made from shelled pecans.

Pecan meal was leached with water before being incorporated in the diet of Rats 57 and 58.

The integument of the pecan nuts used in the ration of Rat 44 was partly removed by scraping each nut with a knife.

The composition of these diets was the same as described in Chart 1.

The difference in growth of the two lots is a striking example of the effect that some distasteful or injurious substance in the food may have on the growth of rats. Unless the components of a ration are carefully scrutinized for the presence of substances that may render the diet unfit for the animals for which it is prepared, there is always danger of incorrectly explaining the response of animals to such diets. The care that must be taken in interpreting the cause of failure of feeding experiments cannot be emphasized too often.

SUMMARY AND CONCLUSIONS.

The principal protein of the pecan nut is a globulin. This globulin has been isolated and the distribution of its nitrogen determined by the Van Slyke method.

Normal growth has been observed in young rats whose dietary protein was derived from the pecan nut, indicating that the nut is a source of adequate protein.

The presence of tannins in pecan diets has been shown to be a limiting factor for the growth of rats.

STUDIES ON EXPERIMENTAL RICKETS.

IX. LESIONS IN THE BONES OF RATS SUFFERING FROM UNCOMPLICATED BERI-BERI.

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PLATES 1 TO 4.

(Received for publication, October 11, 1921.)

As far as we are aware no investigators have reported observations on bone changes produced in animals by diets so constituted as to cause uncomplicated beri-beri or polyneuritis.

In studying the bones of rats suffering from polyneuritis induced by diets deficient in the water-soluble B we were surprised to find that in practically all histological details they were exactly like those of guinea pigs suffering from uncomplicated scurvy induced by a diet consisting of soy beans (cooked) 60.0, casein 10.0, wheat germ 10.0, maize 12.0, NaCl 1.0, $CaCO_3$ 2.0, butter fat 3.0, and cod liver oil 2.0 (Figs. 3 to 6). On this diet, which is complete except for the absence of the antiscorbutic substance, guinea pigs developed acute scurvy in about 3 weeks. When small amounts of orange juice or other antiscorbutic food are added to it they remain free from scurvy. These results were so unlooked for that we report preliminary observations on the bones of these rats. Further studies are in progress to determine the limits of dietary conditions which can induce such changes.

Polyneuritis was brought on in young rats by feeding a diet consisting essentially of purified food substances, and lacking (for the nutrition of the rat) only in the antineuritic substance, water-soluble B. The food mixture was constituted as follows:

Ration 3106.

	per cent
Casein	18.0
Salt mixture (185)	3.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

On this diet the animals usually increased in weight during the first 3 weeks, then began to decline. After a few weeks (40 to 60 days) they usually developed the symptoms characteristic of beri-beri, although some animals died without developing the acute symptoms of the disease. (See Chart 1, Lot 3106 and Figs. 1 and 2.)

At autopsy all the body tissues of the rats were atrophic. There was no deformity of the skeleton and no noticeable enlargement of the extremities of the bones.

The picture of these bones through the microscope was quite indistinguishable from that seen in the bones of guinea pigs with scurvy (Figs. 3 and 4). The bones were very osteoporotic. One received the impression that all growth must have stopped. The epiphyseal cartilage was very shallow (only 4 to 5 cells in depth) and stained intensely with basic dyes. This was true of the cell bodies as well as the matrix. The cells in the narrow proliferative zone were very much flattened in the direction of the long axis of the bones. The intracellular substance of the cartilage was very abundant, and in the shallow zone of provisional calcification it was heavily infiltrated with lime salts. Here and there at intervals thick, heavily calcified rods of intracellular substance projected from the medullary border of the cartilage into the marrow cavity which was elsewhere in contact with the calcified provisional zone. Many of these projecting spicules of intracellular substance were rounded off and were capped by multinucleated masses of protoplasm which were apparently osteoclasts. The cortex of the shaft was of fair thickness and was solid and completely calcified. The marrow contained comparatively few nucleated cells (leucocytes and their predecessors). The blood vessels of the medullary cavity were crowded with red blood cells. In many places the vessels had ruptured and numerous small and large hemorrhages were to be found, especially in the vicinity

of the cartilage (Fig. 5). In some bones the active bone marrow had been almost entirely replaced by these hemorrhages. Here and there at wide intervals small thick trabeculæ or trabecular remnants were to be found scattered through the hemorrhagic area which represents the marrow. These were completely calcified and were surrounded by an endosteum which consisted of a broken layer of osteoblasts, and a fine fibrillar network filled with mononuclear cells.

The destruction of the marrow elements is not the result of hemorrhage. The hematopoietic marrow may entirely disappear before hemorrhage has occurred and practically no trace of it remains except the supporting reticulum (Fig. 7). The medullary cavity under these conditions contains nothing but a cellular network of fine reticular tissue supporting numbers of thin walled blood vessels filled to capacity with corpuscles.

It may very well be that the dilatation and weakening of the blood vessels of the marrow and their consequent solution or rupture in the bones of rats on the diet low in water-soluble B is analogous to the condition of the intestine described by McCarrison (1). It is quite as likely that the vessel wall is directly affected by deprivation of water-soluble B as that the lesion is secondary to an affection of the nerve supply to the medullary vessels. The condition of the marrow is extremely interesting in the light of the findings of Happ,[1] who has studied the blood of rats in polyneuritis. He found that the number of red cells and the hemoglobin percentage was unchanged in the circulating blood. His animals showed, however, a marked leucopenia, with the white cells in one case as low as 1,800 per c. mm. (the normal white count for the rat is 10,000 cells per c. mm.). There was also a relative reduction in the number of polymorphonuclear cells and a marked shift to the right of Arneth's formula. This condition corresponds to the blood picture in human cases of beri-beri as reported by Findlay (2). Taken in conjunction with the condition of the bone marrow these findings can only indicate a markedly diminished hematopoiesis with abnormal conservation of the formed elements already in the circulation.

[1] Happ, W., personal communication. Dr. Happ's paper on the blood of rats which have been fed faulty diets is in press and will be published shortly.

Rats were fed on the same diet as the guinea pigs above described. Their growth is illustrated by the curves in Chart 2. Their bones were examined after they had been restricted to this scorbutic diet for 19 and 105 days. On this diet female rats not only grew to weights of 240 gm., but produced large litters of young (8, 10, and 12) and successfully weaned them. The young were not in an optimal condition but grew on the family diet for months and reached body weights of 200 gm. These results leave no room for doubt that the rat can dispense with water-soluble C provided the rest of the diet is satisfactory.

Examination of the bones of these rats revealed no evidence of departure from the normal in histological structure (Fig. 8).

The studies of McCollum and Simmonds some years ago (3) showed that rats may subsist and grow well for many months on diets free from the antiscorbutic substance, and show no evidence of developing scurvy. Parsons (4) showed that livers of rats long deprived of the antiscorbutic factor will promptly cure scorbutic guinea pigs. The prairie dog has also been shown to be capable of growth and long maintenance on a diet which will promptly induce scurvy in guinea pigs (5).

The growth and state of nutrition of another group of rats restricted to this food mixture (Lot 2983, Chart 1) supplemented with 30 per cent of rolled oats which served to supply an abundance of water-soluble B also show that there was no known defect in our diet for the rat other than lack of the dietary factor responsible for beri-beri; *i.e.*, water-soluble B, Chart 1 (6). Oats contain neither fat-soluble A nor water-soluble C in demonstrable amounts. This purified food mixture supplemented with 3 per cent of wheat germ, or with an extract of wheat germ, is likewise capable of inducing growth (see Chart 3). A similar mixture of purified foodstuffs (with butter fat) and supplemented with 25 per cent of cooked navy beans also induces good growth and fertility in the rat (see Chart 4). It will be evident, therefore, that the rats on Formula 3106 were fed a diet which was complete for the rat except for the lack of antiberi-beri substance, water-soluble B.

The bones of rats fed the same diet with 30 per cent of rolled oats were studied histologically. These revealed no lesions whatever, notwithstanding the specific fasting of these rats for the antiscorbutic substance, water-soluble C. Tozer (7) claimed in a

foot-note to a paper describing the lesions in the bones of scorbutic guinea pigs that a deficiency of the fat-soluble A in the diets of these animals may cause changes "indistinguishable from those termed incipient and definite scurvy." The bones of rats on diets low in this substance alone are osteoporotic (Chart 1, Lot 2031). They have no changes resembling the lesions of scurvy. The bone marrow is quite normal and no hemorrhage or congestion is present.

CONCLUSIONS.

1. Rats fed a diet complete except for the absence of the anti-beri-beri factor develop lesions in the bones which are essentially identical with those seen in guinea pigs suffering from acute and uncomplicated scurvy. Rats confined to the same diet supplemented with water-soluble B do not show these changes.

2. The bones of rats on a diet which is only deficient in the fat-soluble A are osteoporotic but have no other resemblance to the bones of scorbutic animals.

Chart 1, Lot 3106. These rats were restricted to a diet which was adequate in all respects except that it lacked entirely the antineuritic substance, water-soluble B. The animals were unable to grow because of this deficiency and in many cases developed the typical symptoms of polyneuritis.

The bones of these animals were not deformed as in rickets, but were osteoporotic and exhibited characteristic hemorrhages under the microscope. These peculiarities have been described in the text of this paper. They were indistinguishable from the bones of guinea pigs which were suffering from acute scurvy.

Lot 2983 had a diet essentially similar in all its properties to that of the animals just described (Lot 3106) except that the *antineuritic substance* was added in the form of 30 per cent of rolled oats. Oats contain no antiscorbutic factor and no demonstrable amount of fat-soluble A. The latter factor was furnished to both groups (Lots 2983 and 3106) in the form of butter fat. While the animals did not grow at the maximum rate, nor reach the maximum adult size, they were fairly well nourished and were fertile. They destroyed almost all their young soon after they were born.

This diet, entirely free from antiscorbutic substance, but containing the antineuritic factor, induced the development of entirely normal bones. There seems to be no other interpretation warranted than that the specific bone changes found in Lot 3106 were due to lack of water-soluble B in the diet.

Lot 2031 is included here for the sake of completeness. It contained in the rolled oats a sufficient amount of water-soluble B, as is shown by the growth and reproduction of Lot 2983. It was entirely deficient in the

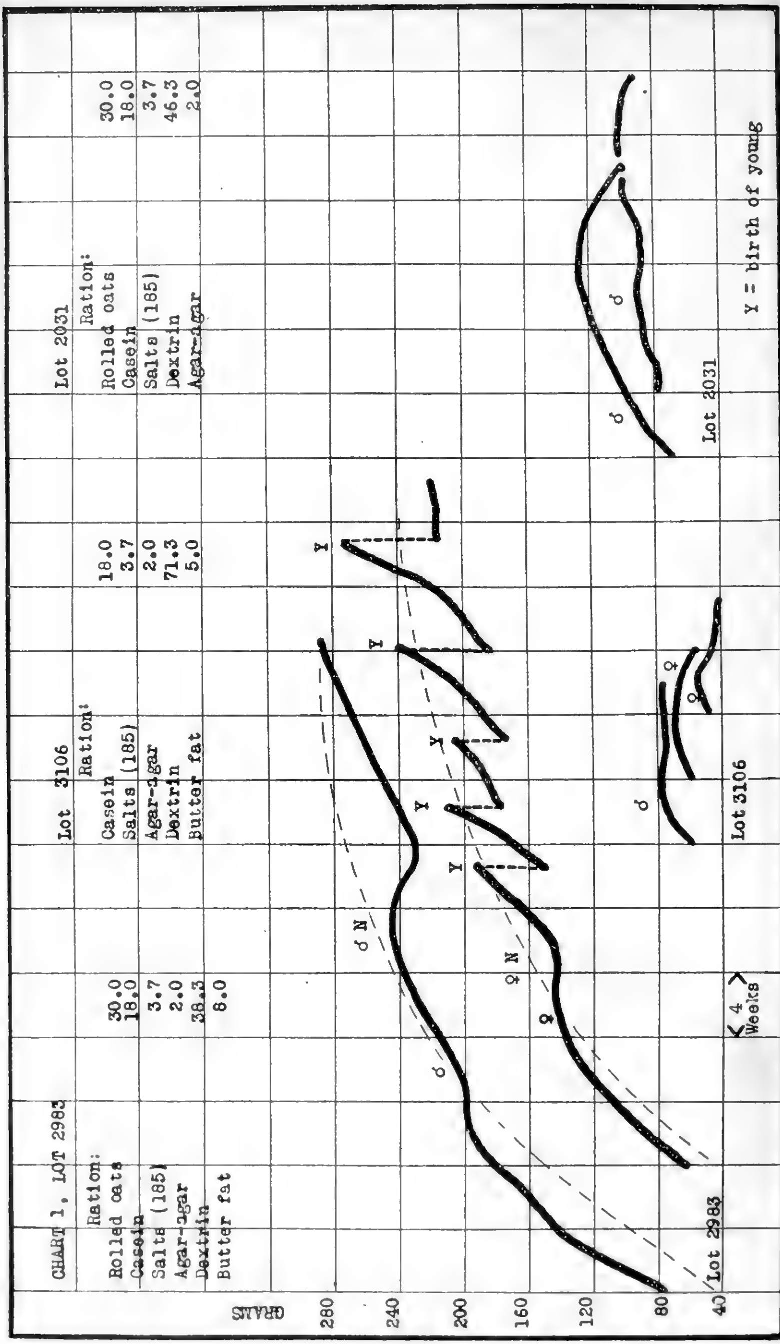
CHART 1. LOT 2983
Ration:
Rolled oats 30.0
Casein 18.0
Salts (185) 3.7
Agar-agar 2.0
Dextrin 38.3
Butter fat 8.0
Lot 3106
Ration:
Casein 18.0
Salts (185) 3.7
Agar-agar 2.0
Dextrin 71.3
Butter fat 5.0
Lot 2031
Ration:
Rolled oats 30.0
Casein 18.0
Salts (185) 3.7
Dextrin 46.3
Agar-agar 2.0
GRAMS
280
240
200
160
120
80
40
4 Weeks
♂ N
♀ N
Y
Lot 2983
Lot 3106
Lot 2031
Y = birth of young

CHART 1.

antiscorbutic substance and, because of the omission of butter fat from the formula, the diet was essentially free from fat-soluble A. Since all the evidence supports the view that the antiscorbutic substance is dispensable from the diet of the rat the diet of this group was faulty only in respect to lack of fat-soluble A. The addition of butter fat (fat-soluble A) makes the food mixture like that of Lot 2983, which was complete.

Lack of fat-soluble A did not in these experiments induce changes in the bones which in any way resembled those described and attributed in the rat to beri-beri and in the guinea pig to scurvy. The bones were osteoporotic (8). With the addition of butter fat the diet became capable of supporting normal bone development.

We conclude from these studies that *in the rat* restricted to a diet of purified food substances (free from all vitamines) the addition of fat-soluble A alone induces bone changes identical with those seen in scurvy in guinea pigs, whereas the addition of water-soluble B (in the absence of fat-soluble A) induces osteoporosis. It is further to be noted in agreement with earlier studies that diets containing satisfactory amounts of calcium and phosphorus do not produce rickets even when nearly free from fat-soluble A (8).

Chart 2, Lot 3168. These rats were restricted throughout the entire growth period and later to a diet which induces severe scurvy in the guinea pig within 2 to 4 weeks. Rats are, however, able to grow rapidly to the full adult size, exhibit the normal fertility, and rear their young with a low mortality rate. The second generation was able to grow to maturity and reproduce and rear young. There can be no question that the rat may be satisfactorily nourished without a supply of the antiscorbutic substance, water-soluble C.

The histological picture of bone sections from these animals showed the normal structure.

Chart 3, Lot 2089. This diet was essentially comparable to that of Lot 3106 (Chart 1) except that it contained water-soluble B derived from the alcoholic extract of 10 gm. of wheat germ per 100 gm. of ration. This induced essentially normal growth, although the animals remained somewhat undersized. These animals were produced some years ago in our laboratory and their bones were not examined. We know, however, that rats which were able to grow as well as these did would have bones with no serious defects.

Lot 2088 was fed a diet of purified food substances completed qualitatively by the addition of fat-soluble A and water-soluble B in 4 per cent of butter fat and 3 per cent of wheat germ respectively. Fair growth was possible and one female had three litters of young.

Chart 4, Lot 696. These rats were fed a diet derived in great measure of purified food substances, but supplemented with water-soluble B in the form of 25 per cent of cooked navy beans, and fat-soluble A in 5 per cent of butter fat. This diet contained no antiscorbutic substance, yet young rats were able to grow rapidly to full adult size when restricted to it. Even the second generation of rats on this diet grew fairly well.

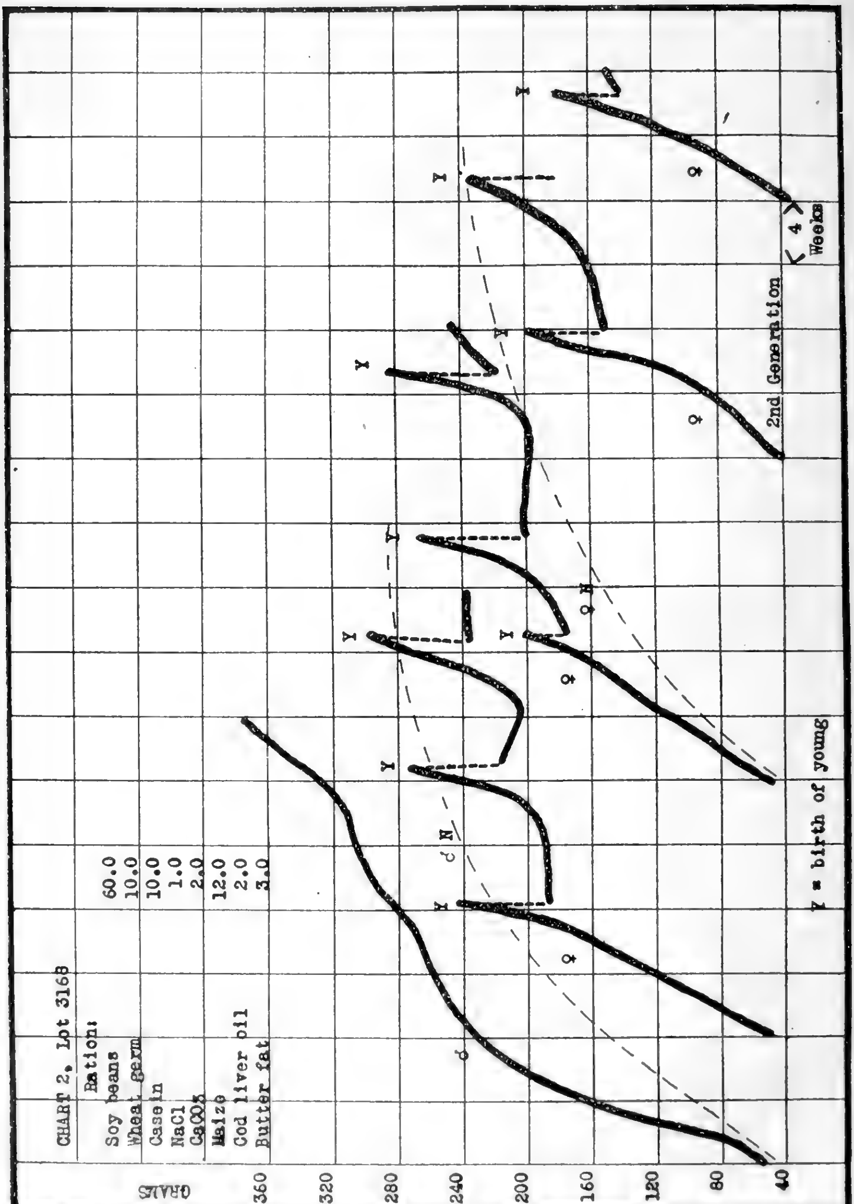
CHART 2. Lot 3168
Ration:
Soy beans 60.0
Wheat germ 10.0
Casein 10.0
NaCl 1.0
CaCO3 2.0
Maize 12.0
Cod liver oil 2.0
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
Y = birth of young
2nd Generation
4 Weeks

CHART 2.

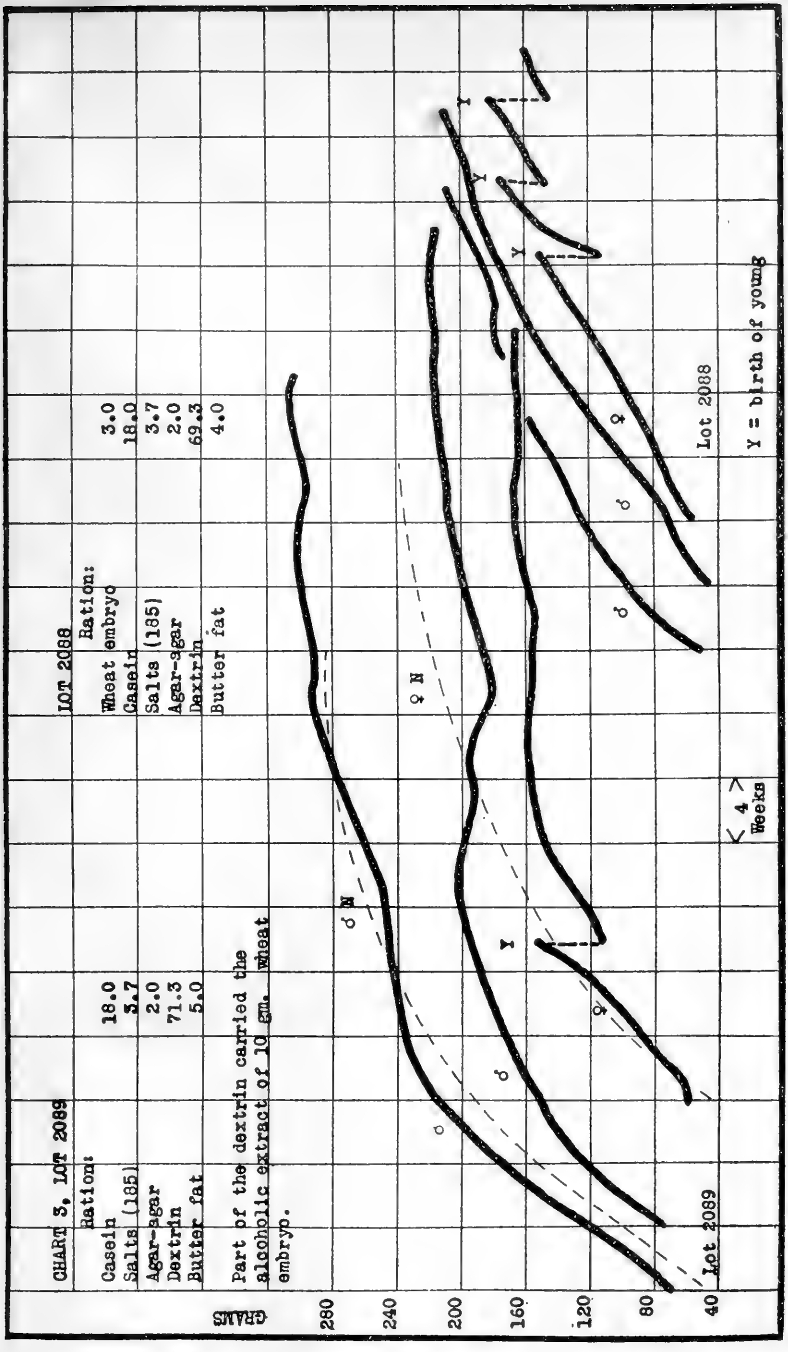
CHART 3, LOT 2089
Ration:
Casein 18.0
Salts (185) 3.7
Agar-agar 2.0
Dextrin 71.3
Butter fat 5.0
Part of the dextrin carried the alcoholic extract of 10 gm. wheat embryo.
LOT 2088
Ration:
Wheat embryo 3.0
Casein 18.0
Salts (185) 3.7
Agar-agar 2.0
Dextrin 69.3
Butter fat 4.0
GRAMS
280
240
200
160
120
80
40
Lot 2089
Lot 2088
♂ N
♀ N
Y
♂
♀
< 4 >
Weeks
Y = birth of young

CHART 3.

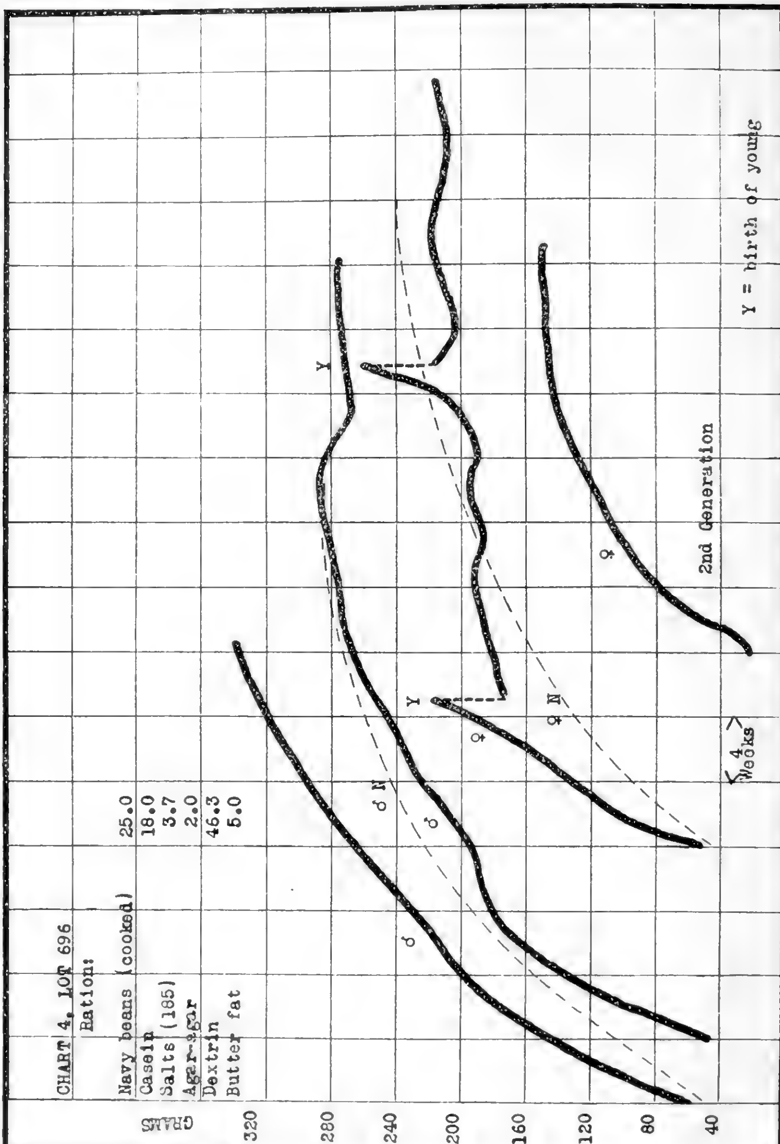
CHART 4, LOT 696
Ration:
Navy beans (cooked) 25.0
Casein 18.0
Salts (185) 3.7
Agar-agar 2.0
Dextrin 46.3
Butter fat 5.0
GRAMS
320
280
240
200
160
120
80
40
♂
♂
♂ N
♀
♀ N
♀
Y
Y
4 Weeks
2nd Generation
Y = birth of young

CHART 4.

These several groups of growth curves of rats are presented in order to illustrate how effectively a diet of purified food substances can be supplemented by means of additions which do not contain demonstrable amounts of the antiscorbutic substance. They form a complete demonstration of the fact that the bone changes induced in those rats which were selectively fasted for water-soluble B only were due to lack of this dietary essential.

It has been pointed out by Vedder (9), Hess (10), and others, that there are similar nervous manifestations and pathological changes in the heart in human cases of scurvy and beri-beri. Unfortunately, one cannot place much confidence in the description of these diseases as they occur in man, for an examination of the diets on which people develop either of these diseases makes it practically certain that beri-beri scarcely ever occurs except in an individual who is a border-line case of scurvy. Scurvy doubtless has, however, occurred many times without complication with beriberi. We have no assurance that any description of histological studies of any human tissues were made on subjects suffering from one of these diseases uncomplicated with the other.

Nevertheless there is an added interest, in the repeated statements of others that there are lesions in beri-beri and scurvy which are common to the two diseases. Our observations show that the histological changes in the bones of rats suffering from uncomplicated beri-beri are apparently identical with those seen in the guinea pig suffering from uncomplicated scurvy.

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EXPLANATION OF PLATES.

Plate 1.

Figs. 1 and 2. This rat was in a condition of polyneuritis induced by feeding Diet 3106 for 70 days. Fig. 1 shows the animal rising to his feet from the spread-eagle posture assumed during rest in a ventral position (Fig. 2). Note the spasticity and the extreme extension and abduction of all four extremities, the extension of the digits, and the roughness of the coat.

Plate 2.

Fig. 3. Section of a long bone from a guinea pig with acute scurvy. The bone is well calcified throughout but is extremely osteoporotic. The marrow cavity shows numerous hemorrhages. *H* = areas of hemorrhage. Leitz microsummar. 35 mm. objective. No ocular.

Fig. 4. Long bone of a rat killed during an attack of polyneuritis. This bone was osteoporotic and the hematopoietic marrow was almost entirely replaced by hemorrhage. *H* = areas of hemorrhage. Same magnification as Fig. 3.

Plate 3.

Fig. 5. High power picture of the marrow cavity of the bone shown in Fig. 3 to show the hemorrhagic marrow. *H* = areas of hemorrhage. Leitz objective No. 6. No ocular.

Fig. 6. To show the replacement of the bone marrow by hemorrhages during polyneuritis. *H* = hemorrhage. Magnification as in Fig. 5.

Plate 4.

Fig. 7. Bone marrow of a polyneuritic animal during the congestion stage before hemorrhage has occurred. The marrow consists only of reticular tissue supporting widely dilated congested blood vessels. *M* = marrow. Same magnification as in Fig. 5.

Fig. 8. To show the bone of a rat which was fed on the diet which we used to induce scurvy in our guinea pigs. This bone is quite normal.

Fig. 1.

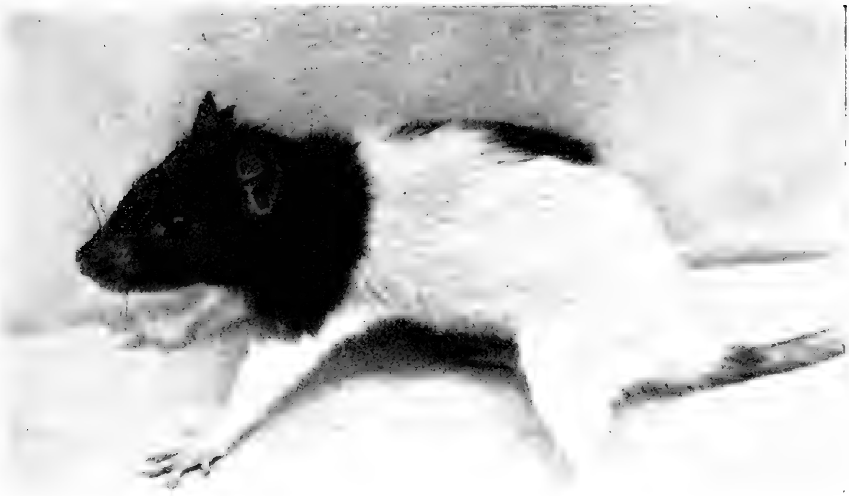

Fig. 2.

(Shipley, McCollum, and Simmonds: Studies on experimental rickets. IX.)

Fig. 3.

Fig. 4.

(Shipley, McCollum, and Simmonds: Studies on experimental rickets. IX.)

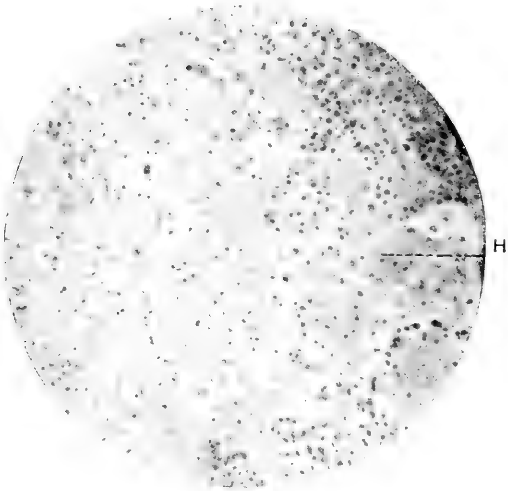

FIG. 5.

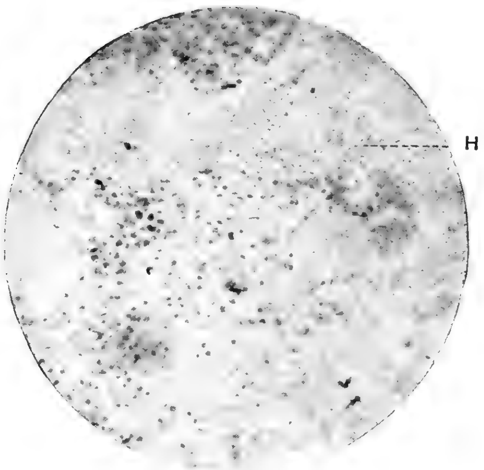

FIG. 6.

(Shipley, McCollum, and Simmonds: Studies on experimental rickets. IX.)

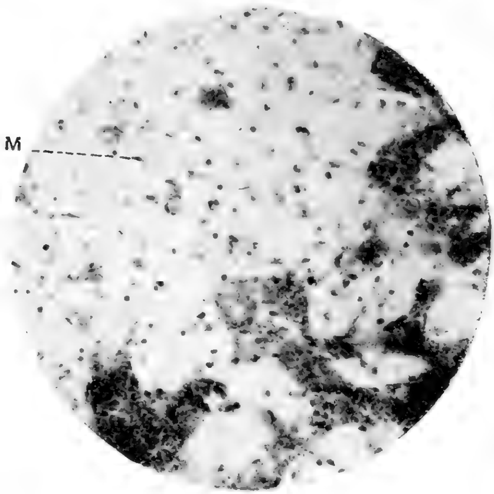

Fig. 7.

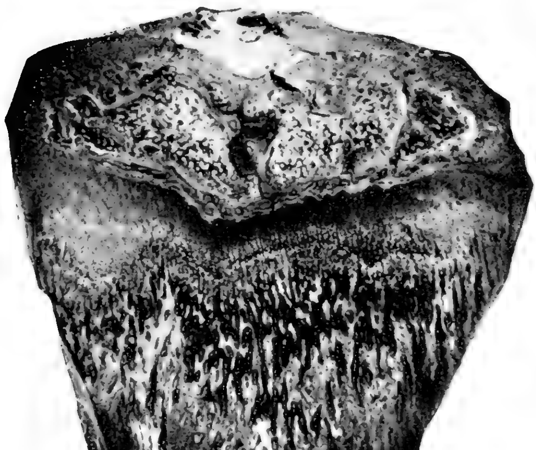

Fig. 8.

(Shipley, McCollum, and Simmonds: Studies on experimental rickets. IX.)

AMMONIA EXCRETION FOLLOWING EXPERIMENTAL ADMINISTRATION OF ACIDS VIA THE STOMACH AND PERIPHERAL VEIN.

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The type of acidosis which attains its highest expression in diabetes is, as is well known, very frequently attended by a marked increase in the excretion of ammonia in the urine. The acids in this case (β-hydroxybutyric and acetoacetic) are in part neutralized by the organic basic ammonium. Yet the phenomenon of neutralization by ammonium is not constant in the other types of acidosis in man. It appears to play relatively a small part in the acidosis of uremia (1), even when this attains an extreme degree, and it is missed in some of the other types of spontaneous acidosis, which have been observed in man. On the other hand, the feeding of dilute mineral acids (2, 3) to man, if in sufficient quantity, leads consistently to increased excretions of ammonia in the urine comparable to those seen in the diabetic type of acidosis. However, if a man, who is excreting increased quantities of ammonia, either as an incident in the course of a spontaneous diabetic acidosis or as the result of an alimentary administration of an acid such as hydrochloric, be given a sufficient quantity of bicarbonate of sodium (4), the increased excretion of ammonia is depressed or wholly annulled. The administration of the stronger base results in the displacement of the excess of ammonia from the urine, and it would not seem irrational to compare the phenomenon with that which occurs in the test-tube, when sodium bicarbonate, magnesium oxide, or calcium oxide, are added to a watery solution of ammonium chloride, acetoacetate, β-hydroxybutyrate, or the like with the liberation of free ammonia and the formation of a new salt of the base employed. In the body such a release

of ammonia from its salts (or the prevention of its union with acids) by the action of a stronger base could be conceived as leading simply to some other disposition of the ammonium radicles displaced or spared from combination with acids. They might then, for example, enter into the synthesis of urea.

Although the phenomenon of neutralization of acids by ammonia has long attracted interest and has been spoken of by some writers (5) as one of the important compensatory mechanisms by which the body may protect itself against inroads of acid, it is a noteworthy fact, that it is not a mechanism which is called into play consistently by the human organism under all conditions, in which it might be supposed in reason that every available means of protection should be employed. A patient may die of acid intoxication developing in the course of a pyelonephritis without ever having called on this resource to a noticeable degree. In man the classical examples of neutralization of acids by ammonia are afforded in acidosis of the diabetic type. It has been reported in clinical methyl alcohol poisoning (6) which, however, under experimental conditions (7) may not always be associated with an acidosis. It is also said to develop in the acidosis of cholera (1) and it has been shown to occur in connection with certain of the diarrheal diseases of children, which in some cases, resemble closely the acidosis of diabetes. The two chief conditions, however, in which the phenomenon is known to occur regularly are those mentioned. Moreover, this type of acidosis associated with increased ammonia output is not seen in all types of animals but occurs most readily in man and the carnivorous animals, and only to a lesser extent in the herbivorous animals. These considerations suggest that the neutralization of acids by ammonium is not a mechanism of universal significance in the whole problem of neutrality regulation by living cells, but that it is rather a special phenomenon which only occurs under certain conditions.

It has been pointed out by others (8) that, in discussing observed variations in the excretion of acetone bodies in the urine, it is pertinent to consider the question of where the acetone bodies are formed. Well known evidence afforded by liver perfusion experiments, favors the view, that the liver, of all the organs, may be regarded as the largest single producer of the acetone bodies. The

liver is also commonly recognized as an organ in which the total traffic in NH_2 groups is greater than in any single tissue, even though the function of deamination and the synthesis of urea were performed to some extent elsewhere as well.[1] With its deaminizing and urea-forming function it must be considered that the liver is preeminent in respect to the total quantity of ammonia equivalents that it normally handles in the course of the day. It is generally accepted that the ammonia which in acidosis appears in the urine in the form of salts of acids represents basic nitrogenous material, which normally would be excreted for the most part in the form of urea. Also acids, which are formed and liberated in this organ which conducts the heaviest traffic in ammonia or its equivalents, are generated in a part of the body where the chances for neutralization by ammonia should be exceptionally favorable.

If it be granted that the phenomenon of neutralization of acids by ammonium has been observed with the greatest consistency and in the highest degree in two conditions: (*a*) a type of acidosis in which the acids are largely hepatogenous in origin, and (*b*) a type following the administration of acids—but that the phenomenon is found to be less marked or entirely absent in other types of acidosis the question would arise as to why the ingestion of acid should produce an effect like that of one type of acidosis and unlike that of the other types. Could this mean that in order to be neutralized to the highest degree by ammonium the free acids involved must be formed within or be introduced into the liver?

In reviewing the literature one is struck by the fact that those who have observed increased excretion of ammonia following the administration of acids have almost without exception given the acid by the alimentary route. Winterberg (10) injected rabbits subcutaneously (two experiments) with 0.25 N acid and found the ammonia, sodium, and potassium content of the urine greatly

[1] Stadie and Van Slyke (9) in their study of a case of acute yellow atrophy have recently concluded that the rôle of the liver in these processes cannot be assumed in its entirety by the rest of the body. Only in pathological conditions, which are associated with almost complete destruction of the liver tissue, does the organism fail to metabolize in a normal fashion the amino-acids even though they be presented in high concentrations.

increased. The total nitrogen was not correspondingly increased. But one of these animals died at the end of 24 hours and with the other the experiment was discontinued in about the same time. These two isolated experiments would scarcely seem sufficient to settle the point. One would have to consider that the local destructive effect on the tissues of an amount of 0.25 N acid sufficient to cause death in 24 hours is not to be neglected. Whereas, the literature contains other reports (11, 12, 13) concerning the injection of acids by vein we have not found any in which the behavior of the urinary ammonia was recorded.

The experiments to be described were undertaken for the specific purpose of comparing the relative effects on the ammonia excretion in the urine of acid administration by the alimentary route and by the peripheral vein. Alimentary administrations should subject the liver to the effects of a relatively large quantity of free acid, while the latter would afford opportunity for the neutralization of more of the acid in the blood or tissues outside of the liver.

EXPERIMENTAL.

Methods.

Female dogs of approximately 16 kilos body weight were used. They were fed a constant diet, consisting of milk 100 to 200 cc., bread 50 to 100 gm., sodium acetate 0.5 gm. twice daily at 6.30 a.m. and p.m. To the morning feeding 100 cc. of physiological salt solution were added, which served to keep the volume of liquids the same in the experimental and the control periods. The sodium acetate was given to promote uniform elimination. Collections of urine were made twice daily by catheterization and irrigation of the bladder. The cages were scrubbed and disinfected every 12 hours to prevent decomposition of occasional specimens passed spontaneously. This procedure gave consistent ammonia values. The urines were placed on ice without preservatives, the ammonia and total nitrogen being estimated shortly after the closure of the 24 hour period.

The ammonia was estimated by means of formalin titration (Ronchese and Malfatti) as described by Wiechowski (14). The urine was well diluted and the titration was carried to the first

permanent pink. This method was checked with Folin's (15) aeration method. The practical agreement of the methods for present purposes is shown in the following protocol.

Formalin titration.	Folin method.
0.30	0.27
0.25	0.25
0.37	0.37
180 cc. of 0.1 N HCl by stomach.	
0.41	0.41
0.46	0.44
0.39	0.39
0.37	0.38
0.42	Not estimated.
250 cc. of 0.1 N HCl by vein.	
0.46	0.45
0.51	0.51
0.52	Not estimated.
0.33	0.33
0.49	0.48
0.44	0.42

The total nitrogen was estimated by the Kjeldahl method, the creatinine according to Folin (15).

On the morning of the experiment the salt solution was omitted from the diet. The acid was then administered either by stomach tube or into the leg vein. For the latter a cannula was inserted into the vein under local anesthesia, and the acid was introduced by gravity throughout the period of 1 hour.

Experiments.—Five animals were used in the studies, and seven complete (gastric and intravenous administration) experiments made, but the protocols of only two animals are reported in detail. Dog 2 died during the course of the intravenous injection, the time of which was shortened from 1 hour to 30 minutes. Dog 3 was a young, highly irritable animal, which never gave consistent results during the control period. However, the results in this case were in keeping with those reported. The dose of acid injected into Dog 4 proved to be too toxic, so that at the end of 24

hours food was refused, the nose was hot, and the quantity of urine was much reduced. However, the output of ammonia and total nitrogen during the first 24 hours was the same as for the day preceding the experiment, in agreement with the findings to be reported in detail. On the succeeding days the total nitrogen

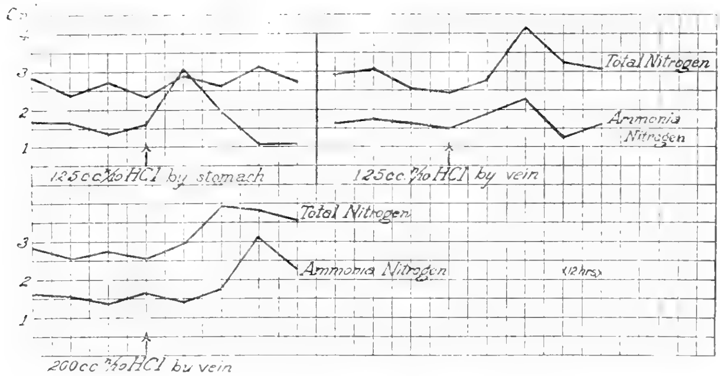

CHART 1. Changes in nitrogen excretion after acid administration in Dog 1.

In the ordinates of Charts 1 to 4 are expressed the values of total and ammonia nitrogen in grams, the latter multiplied by 10. In the abscissæ two spaces represent 12 hours. Record covers 48 hours before and after experiment.

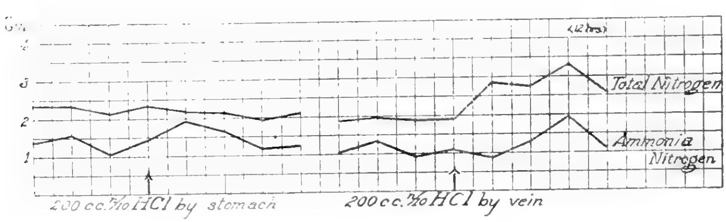

CHART 2. Changes in nitrogen excretion after acid administration in Dog 1.

fell slightly below normal, but the ammonia increased. The results of the experiments on Dogs 1 and 5 are presented in the charts. In Charts 1 to 4 the quantitative output of ammonia nitrogen and the total nitrogen, expressed in grams, are plotted for 48 hours before and after the experimental periods. Chart 5

shows the complete record of Dog 1 through two consecutive experiments. The nitrogen values here are expressed in 24 hour intervals. The figures from which the charts were compiled are to be found in Tables I to V, Chart 1 corresponding to Table I, etc.

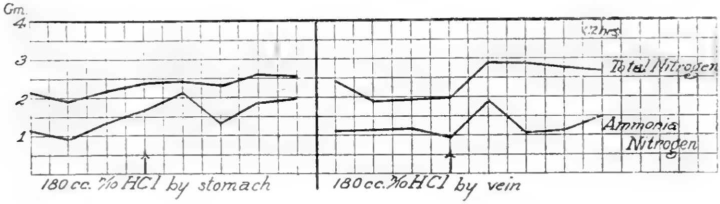

CHART 3. Changes in nitrogen excretion after acid administration in Dog 5.

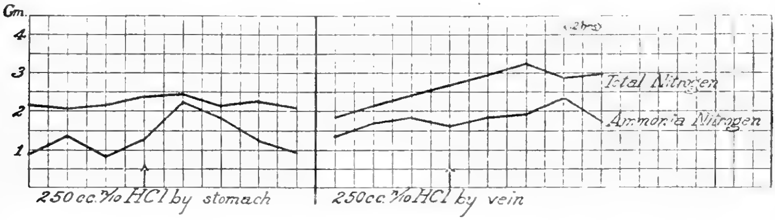

CHART 4. Changes in nitrogen excretion after acid administration in Dog 5.

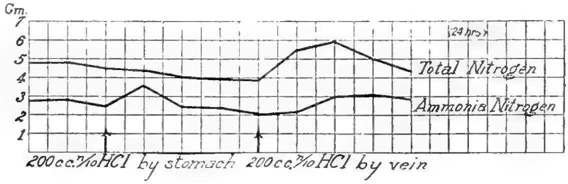

CHART 5. Changes in nitrogen excretion following successive acid administrations in Dog 1.

In the ordinates of Chart 5 are expressed the values of total and ammonia nitrogen in grams, the latter multiplied by 10. In the abscissæ two spaces represent 24 hours. All of the time (11 days) which elapsed in the course of the experiment is represented in the chart.

Ammonia Excretion Following Administration of Acid by Stomach.—These graphs show clearly an absolute increase in the ammonia nitrogen without changes in the total nitrogen. In

other words, some of the nitrogen which normally appeared in the urine in other forms (*e.g.*, urea) has been diverted to the neutralization of the acid and now appears as ammonia in the urine. This simply confirms commonly accepted facts.

Ammonia Excretion Following Administration of Acid Intravenously.—The curve of total nitrogen gradually rises. The suddenness of the rise appears to depend within limits on the quantity

TABLE I.

Changes in Nitrogen Excretion after Acid Administration in Dog 1.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1921	*gm.*	*gm.*
Jan. 18, p.m.	2.86	0.17
" 19, a.m.	2.43	0.17
" 19, p.m.	2.71	0.14
" 20, a.m.	2.35	0.16
" 20, "	Gastric administration 125 cc. of 0.1 N HCl.	
" 20, p.m.	2.93	0.30
" 21, a.m.	2.64	0.20
" 21, p.m.	3.03	0.16
" 22, a.m.	2.70	0.15
Jan. 24, p.m.	2.97	0.17
" 25, a.m.	3.03	0.18
" 25, p.m.	2.51	0.16
" 26, a.m.	2.46	0.15
" 26, "	Intravenous administration 125 cc. of 0.1 N HCl.	
" 26, p.m.	2.79	0.19
" 27, a.m.	4.14	0.29
" 27, p.m.	3.25	0.13
" 28, a.m.	3.06	0.21

of acid given. The ammonia nitrogen follows closely, so that the percentage relationships between ammonia nitrogen and total nitrogen are fairly maintained. This is interpreted to mean that there is a definite toxic destruction of protein induced by the acid injection. The results of these experiments differ from the foregoing in the maintenance of a practically normal percentage of ammonia nitrogen, which suggest simply an acceleration of normal proteolysis.

Comparison.—Tables VI and VII contrast the reactions of the body to acid administration by different routes. The values of total and ammonia nitrogen actually excreted are found in Columns a and b. The first reading of each column in the various experiments gives the excretion during the control period, the second figure, the excretion during the experimental period (24 hours following acid administration). To secure the values for

TABLE II.

Changes in Nitrogen Excretion after Acid Administration in Dog 1.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1920	*gm.*	*gm.*
Dec. 8, p.m.	2.38	0.13
" 9, a.m.	2.36	0.15
" 9, p.m.	2.16	0.11
" 10, a.m.	2.33	0.14
" 10, "	Gastric administration 200 cc. of 0.1 N HCl.	
" 10, p.m.	2.21	0.20
" 11, a.m.	2.19	0.17
" 11, p.m.	1.97	0.12
" 12, a.m.	2.08	0.13
Dec. 12, p.m.	1.92	0.11
" 13, a.m.	1.99	0.13
" 13, p.m.	1.93	0.10
" 14, a.m.	1.96	0.11
" 14, "	Intravenous administration 200 cc. of 0.1 N HCl.	
" 14, p.m.	2.85	0.09
" 15, a.m.	2.72	0.13
" 15, p.m.	3.36	0.20
" 16, a.m.	2.56	0.11

the control period the excretions of total and ammonia nitrogen were averaged for three 24 hour intervals preceding the experiment. It has been assumed for the purposes of this tabulation that the *ratio* of ammonia nitrogen to total nitrogen, established in the control period (Column c), was maintained into the acid period except in so far as it was changed by the formation of ammonium salts. That such an assumption can be made without undue violence is seen by reference to preceding data and

discussion. For it was pointed out that the gastric administration of the acid was without effect on the total nitrogen, and that in the administration by peripheral vein, the results suggest an acceleration of normal proteolysis. The ammonia nitrogen then, which is to be expected on such a basis, can be deduced by multiplying this ratio by the total nitrogen actually excreted after acid administration. Such a value is shown in Column d. The dif-

TABLE III.

Changes in Nitrogen Excretion after Acid Administration in Dog 5.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1921	*gm.*	*gm.*
Mar. 1, p.m.	2.14	0.12
" 2, a.m.	1.93	0.09
" 2, p.m.	2.12	0.14
" 3, a.m.	2.34	0.17
" 3, "	Gastric administration 180 cc. of 0.1 N HCl.	
" 3, p.m.	2.42	0.21
" 4, a.m.	2.30	0.13
" 4, p.m.	2.53	0.18
" 5, a.m.	2.51	0.20
Feb. 14, p.m.	2.44	0.11
" 15, a.m.	1.91	0.12
" 15, p.m.	1.97	0.12
" 16, a.m.	1.99	0.10
" 16, "	Intravenous administration 180 cc. of 0.1 N HCl.	
" 16, p.m.	2.86	0.19
" 17, a.m.	2.85	0.11
" 17, p.m.	2.78	0.11
" 18, a.m.	2.67	0.16

ference between this value and the quantity actually excreted (Column b) would indicate the quantity of ammonia concerned in the neutralization of the acid administered. This value has been expressed in cc. of 0.1 N NH_4OH and denominated "Extra ammonia."

It is obvious from the tables that a much larger quantity of the acid administered by the gastrointestinal route can be accounted for as "Extra ammonia," than of that given by the peripheral

TABLE IV.

Changes in Nitrogen Excretion after Acid Administration in Dog 5.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1921	*gm.*	*gm.*
Apr. 3, p.m.	2.14	0.09
" 4, a.m.	2.07	0.14
" 4, p.m.	2.18	0.08
" 5, a.m.	2.35	0.13
" 5, "	Gastric administration 250 cc. of 0.1 N HCl.	
" 5, p.m.	2.41	0.22
" 6, a.m.	2.12	0.18
" 6, p.m.	2.29	0.12
" 7, a.m.	2.09	0.09
Mar. 8, p.m.	1.82	0.14
" 9, a.m.	2.20	0.16
" 9, p.m.	2.37	0.18
" 10, a.m.	2.67	0.17
" 10, "	Intravenous administration 250 cc. of 0.1 N HCl.	
" 10, p.m.	2.94	0.18
" 11, a.m.	3.30	0.20
" 11, p.m.	2.89	0.24
" 12, a.m.	2.94	0.18

TABLE V.

Changes in Nitrogen Excretion Following Successive Acid Administrations in Dog 1.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1920	*gm.*	*gm.*
Dec. 7– 8	4.75	0.28
" 8– 9	4.74	0.29
" 9–10	4.49	0.25
" 10, a.m.	Gastric administration 200 cc. of 0.1 N HCl.	
" 10–11	4.40	0.36
" 11–12	4.05	0.25
" 12–13	3.91	0.24
" 13–14	3.89	0.21
" 14, a.m.	Intravenous administration 200 cc. of 0.1 N HCl.	
" 14–15	5.57	0.22
" 15–16	5.92	0.30
" 16–17	5.06	0.31
" 17–18	4.30	0.28

TABLE VI.

Quantity of Acid Excreted in the Form of Ammonium Salts in the 24 Hour Urine before and after the Administration of Hydrochloric Acid by the Gastric and Intravenous Routes.

(Dog 1, female mongrel, weight 16.17 kilos.)

Experiment.	0.1 N acid given.		Nitrogen excretion per 24 hrs.				Extra ammonia as 0.1 N NH_4.†	Remarks.
	Amount.	Route.	Total.	In NH_3.	Ratio $\frac{b}{a}$.	Ratio $\frac{b}{a}xy$*.		
			a	b	c	d		
	cc.		gm.	gm.		gm.	cc.	
1			5.43	0.32	0.058			24 hr. control (average of 3 days).
	125	Gastric.	5.57	0.50		0.32	128	24 hr. period following acid.
2			4.66	0.27	0.058			24 hr. control (average of 3 days).
	200	Gastric.	4.40	0.36		0.25	79	24 hr. period following acid.
3			5.63	0.33	0.058			24 hr. control (average of 3 days).
	125	By vein.	6.93	0.42		0.40	14	24 hr. period following acid.
4			3.95	0.23	0.058			24 hr. control (average of 3 days).
	200	By vein.	5.57	0.22		0.32	0	24 hr. period following acid.
5			5.48	0.33	0.060			24 hr. control (average of 3 days).
	200	By vein.	6.94	0.32		0.42	0	24 hr. period following acid.

* The grams of ammonia N which would be excreted if the ratio of ammonia nitrogen to total nitrogen remained the same as in the control period.

† Calculated as the absolute quantity excreted (Column b) minus the above (Column d) and converted into terms of cc. of 0.1 N NH_4 for comparison with the quantity of 0.1 N acid given.

vein. It is also noted that following gastrointestinal administration by no means all of the acid given can be recovered in the

TABLE VII.

Quantity of Acid Excreted in the Form of Ammonium Salts in the 24 Hour Urine before and after the Administration of Hydrochloric Acid by the Gastric and Intravenous Routes.

(Dog 5, female bull, weight 17.8 kilos.)

Experiment.	0.1 N acid given.		Nitrogen excretion per 24 hrs.				Extra ammonia as 0.1 N NH_4.†	Remarks.
	Amount.	Route.	Total. a	In NH_3. b	Ratio $\frac{b}{a}$ c	Ratio $\frac{b}{a} = xy$.* d		
	cc.		gm.	gm.		gm.	cc.	
1			4.41	0.25	0.057			24 hr. control (average of 3 days).
	180	Gastric.	4.72	0.34		0.27	50	24 hr. period following acid.
2			4.35	0.25	0.057			24 hr. control (average of 3 days).
	250	Gastric.	4.53	0.41		0.26	107	24 hr. period following acid.
3			4.10	0.21	0.051			24 hr. control (average of 3 days).
	180	By vein.	5.71	0.30		0.29	7	24 hr. period following acid.
4			4.73	0.32	0.067			24 hr. control (average of 3 days).
	250	By vein.	6.24	0.38		0.42	0	24 hr. period following acid.

* The grams of ammonia N which would be excreted if the ratio of ammonia nitrogen to total nitrogen remained the same as in the control period.

† Calculated as the absolute quantity excreted (Column b) minus the above (Column d) and converted into terms of cc. of 0.1 N NH_4 for comparison with the quantity of 0.1 N acid given.

form of ammonia. This agrees well with the recent report of Stehle and McCarty (3), who found that even with the estimation of all the bases in the urine and the increased H ion concentration

they were able in two cases (man) to account for only 96 and 61 per cent, respectively, of the acid administered.

Creatinine Excretion.—The creatinine excretion was followed in the case of Dog 5 after the administration of 180 cc. of 0.1 N HCl by mouth and 250 cc. of 0.1 N HCl by vein. The variations were so slight as to be within the range of experimental error. We may conclude then that these procedures were without effect on the output of creatine, at least, in this particular animal.

DISCUSSION.

The experiments confirm the well known observation that the administration of acid by the alimentary route to dogs leads to a definite increase in the absolute quantity of ammonia and the quantity relative to the total nitrogen excreted in 24 hours. These increases always took place within the first 24 hours following the acid administration and subsided thereafter. When the same quantity of the same acid was administered to the same dog by the peripheral intravenous route there was as a rule an increase in the total output of ammonia. However, these increases were smaller in degree than those following the alimentary administration, they tended to occur later, and were invariably associated with an increase in the total nitrogen output, so that the ratios of the ammonia nitrogen to the total nitrogen were relatively little affected. Comparing the effects of the alimentary and intravenous administrations of acid, the former were characterized by an increase of the ammonia nitrogen with relatively little increase in total nitrogen. When there was an increase in the total nitrogen at all it was late (3 to 4 days) and never pronounced. In the case of the intravenous injections, the total nitrogen was always increased more and earlier, than in the case of the alimentary administrations. Indeed the speed of the onset of this rise seemed to be determined (within limits) largely by the quantity of acid administered. The total ammonia output then rose correspondingly, but there was little or no evidence of a shift in the nitrogen partition. These results indicate that when the acid enters the body *via* the portal route more of it is neutralized by ammonium than when it enters otherwise.

These results are at variance with those reported by Winterberg (10) to which previous reference has been made. He found that

the ammonia nitrogen was increased without a corresponding increase in the total nitrogen, when rabbits (two experiments) were injected *subcutaneously* with 0.25 N acid. The cause of this discrepancy has not been investigated in detail, but as stated above one must consider that the local destructive action on the tissues of a mass of acid, sufficient to cause death in a rabbit at the end of 24 hours, is not to be neglected. That such a result as he obtained may be associated with the use of extremely toxic or lethal doses of acid is indicated in our findings in Dog 4. The complete details of the experiment are not reported since the animal refused food and showed other signs of profound intoxication. In this case there was no increase in either the total nitrogen or the ammonia nitrogen during the first 24 hours, before the signs of intoxication were manifested and while the animal was still eating. During the 3 succeeding days, with the establishment of these signs of intoxication the ammonia nitrogen increased while the total nitrogen did not increase correspondingly. Finally it must be stated that he was using rabbits while our experiments were conducted upon dogs.

The experiments seem to lend color to the idea developed in the introduction, that neutralization of acids by ammonium may be more in the nature of a localized (hepatic) function than any universal function of the body that is called upon to combat acids in general. It is suggested that when free acids are formed in the liver or are introduced into the liver, and must unite with bases, that in this particular place a larger percentage of the acids will unite with ammonia than will do so when the neutralization takes place elsewhere. Acids must be neutralized by bases that are available at the site of their neutralization. This would imply that in the liver there is ammonium available for the neutralization of acids. The fact that Gad-Andresen (16) and others have shown that the ammonia content of the liver tissue by analysis is not different from that of the heart, muscle, or fat is no argument against this view. For we are here speaking of a moving traffic in ammonia equivalents and not of a cross-section of this traffic at any one time, such as these estimations present.

Such a view would imply that, when endogenous acid appears in the urine in large quantities combined with ammonium, the acids may have been formed chiefly in the liver. Thus diabetes

with its supply of acetone bodies arising chiefly in the liver, presents the classical case of acidosis with large ammonia output. The diarrheas of Asiatic cholera and (less constantly) of infancy with their increased ammonia could be viewed as developing their acids in the portal circuit. Nephritis with its acidosis would then become a disease in which the acids concerned are not hepatogenous in origin, but in which some other principal site or sites of acid formation should be sought. This is not out of harmony with the view that the acidosis of nephritis is due wholly or in part to delayed excretion of acids formed in the body at large. A pathological condition in which the ammonia excretion is increased without a corresponding acidosis would be a condition having no direct interest in the present discussion.

CONCLUSIONS.

1. Administration of 0.1 N HCl by stomach tube to dogs causes an absolute increase in the ammonia nitrogen excreted in the urine, while the total nitrogen remains practically constant.

2. Intravenous administration of the same dose of acid to the same animal causes an increase in excretion of both ammonia and total nitrogen, but the normal ratio between these is fairly well maintained.

3. Alimentary administration of acid is associated with a shift in the nitrogen partition towards the ammonia fraction. Such a shift in nitrogen partition is absent following intravenous injections, if the dose of acid be not too toxic.

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THE COMPOSITION OF CHINESE EDIBLE BIRDS' NESTS AND THE NATURE OF THEIR PROTEINS.*

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Appearance and Origin.

The edible birds' nests are gelatinous substances produced by certain swifts, the *Collacalia*, natives of Malaya (1) and Ceylon. The nests, constructed in caves on the seashore, are collected while they are still moist and made into various shapes. The lowest grade is sold in the form of coarse powder. The higher the grade, the whiter the color and fewer the feathers and twigs. Owing to their high price, their use is limited to a delicacy at the feasts of the wealthy and a food for convalescents and the aged.

The source from which the birds make the nests has been uncertain. Green (2) gives three suggestions: in the algæ found in caves where the swifts make their nests, fish spawn, or a secretion from the swifts themselves. The algæ theory is disproved by the lack of vegetable cells shown by microscopic examination of the nests. The secretion theory is believed by most of the natives and has the support of Home (3) and Bernstein (4). The latter author found in the birds two large salivary glands which secreted much viscous mucus. The observation given in this paper shows that the nests consist largely of a mucin-like substance and, therefore, is in accord with the latter hypothesis.

Review of Literature.

The literature on the subject is limited. Descriptive statements concerning chiefly the occurrence and appearance of the nest may be found in Encyclopedias, China year books, and some semiscientific articles written during the early part of the 19th century. Green (2) and Krukenberg (5) are the first to give a report of a scientific study of the nest. Their work

* The work reported in this article was conducted at the Nutrition Laboratory, Department of Home Economics, University of Chicago. It forms part of the thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Chicago.

covers the solubilities, the response to protein tests, and some observations on hydrolysis products. Their results prove that the birds' nest contains both the carbohydrate and the protein radicle, belonging to the class of mucin-like substances, the glycoproteins.

Scope of the Present Work.

The present work covers the study of the general properties, the chemical composition, the artificial digestion, the carbohydrate radicle, and the biological value of the proteins. Comparison is made with other work on mucin, especially Lothrop's, Müller's, and Levene's.

The material used for this work was supplied by the Hoo Loong Edible Birds' Nest Store, Chicago, which imported it directly from China. It was of the highest grade, having somewhat the appearance of agar-agar, but it was extremely crisp and had tiny feathers interwoven with the mucilaginous material. For quantitative analysis, the material was ground and sifted. On sifting, most of the feathers cling together and may be removed, but some go through the sieve so that it is difficult to obtain a pure sample.

General Properties.

A sample boiled in distilled water for 3 hours and left there for several days, swells like a piece of sponge, but shows no tendency to dissolve. The filtrate responds to neither protein nor carbohydrate tests. 5 per cent sodium hydroxide dissolves it on standing 2 hours in the cold. The colorless solution responds to Millon's, the biuret, xanthoproteic, and Hopkins-Cole tests. The last reaction shows only faintly. It also has a slight reducing power with Fehling's reagent. A dilute acid, such as 3 per cent hydrochloric acid, dissolves the birds' nests only on heating. The solution acquires a purplish brown color, gives both protein and carbohydrate tests, and has a strong reducing action.

So far the properties agree with those reported by Green (2) in every respect except that he found the nest insoluble in dilute sodium hydroxide in the cold. They also agree with the commonly recognized properties of mucin.

Chemical Analysis.

Samples between 1.5 to 2 gm. were taken for the determination of moisture and ash. Neumann's method given in Mathews (6) was used for the estimation of phosphorus, and Denis's method (7) for that of sulfur. An attempt was made to determine the ether-soluble substance, but the results were too small to be of significance, only 0.3 per cent. The estimation of total nitrogen by the Kjeldahl-Gunning method was made on samples treated in three different ways: (1) original birds' nest, (2) ground birds' nest with feathers partially removed, and (3) a sample hydrolyzed for $13\frac{1}{4}$ hours in 20 per cent hydrochloric acid. Results are given in Table I.

TABLE I.

Chemical Composition of Chinese Edible Birds' Nests.

No.	Moisture.	Ash.			Phosphorus.	Sulfur.	Total nitrogen.		
		Water-soluble.	Water-insoluble.	Total.			Original birds' nests.	Ground, and feathers partially removed.	Hydrolyzed $13\frac{1}{4}$ hrs. in 20 per cent HCl.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	11.88	0.75	1.74	2.49	0.035	1.10	8.81	9.16	10.57
2	11.41	0.74	1.80	2.54	0.034	1.10	8.75	9.14	10.24
3	11.52	0.74	1.78	2.52					10.33
4									10.31
Average....	11.60	0.74	1.77	2.51	0.035	1.10	8.78	9.15	10.29

The total ash, 2.51, is almost seven times as high as that of submaxillary mucin, 0.37 per cent, reported by Lothrop (8). This high percentage of ash shows that the birds' nest is not a pure mucin, but more probably dried saliva. Of the total ash 29.48 per cent is insoluble in water, but none insoluble in acid. Hence no sandy material is present.

The sulfur content, 1.10 per cent, is in agreement with the figures given by Müller (9) for salivary mucin, 1.40 per cent, but it is higher than that reported by Lothrop (8), 0.55 per cent. The

discrepancy may be due to the different methods used or in case of birds' nests, the presence of feathers. The phosphorus, 0.035 per cent, is too small to be of any significance.

The different values for nitrogen found in birds' nests treated in three different ways may be explained by the variation in the feathers present. The original sample containing the most feathers, had the lowest figure, 8.78 per cent, while that hydrolyzed with the least feathers, the highest or 10.29 per cent. The ground and sifted sample gave 9.15 per cent. Some feathers were removed during grinding and sifting, but more of them separated out by clinging to the walls of the vessel on hydrolysis. They could then be easily removed. The percentage of nitrogen of the hydrolyzed material, 10.29, agrees with the value given by Müller (9) for salivary mucin, 10.70 for total nitrogen, but it is lower than that reported by Lothrop (8), 12.49 per cent.

Artificial Digestion.

Artificial digestion experiments carried out in comparison with hard boiled egg white showed that the birds' nests were digested by both pepsin hydrochloric acid and trypsin though not so quickly as the egg. The speed of digestion was determined by Sörensen's titration (6). Comparison was made of the increase during 24 hours in the volume of 0.1 N sodium hydroxide for titrating 25 cc. of the peptic digest. Results were expressed in cc. per gm. of nitrogen in the material acted upon. For the birds' nests in a typical experiment this value was 9.60 cc. and for the egg white 15.47 cc. Similarly, the increase of 0.1 N hydrochloric acid to titrate the tryptic digest was 19.02 cc. for the birds' nests and 38.75 cc. for the egg white.

The percentage of carbohydrate in the hydrolyzed birds' nests could not be found with accuracy. Efforts using Benedict's (10) Method 2 failed to give concordant results. The material was prepared in the following manner: 1 gm. of the ground and sifted nests was dissolved in a small amount of concentrated hydrochloric acid by standing over night. It was then diluted with distilled water to make a 5 per cent acid solution and boiled with a reflux condenser for $1\frac{1}{2}$ hours. The hydrolyzed mixture was treated with phosphotungstic acid, filtered, and the filtrate was made up to a definite volume.

Concordant results were not obtained. Variations were therefore made in the strength of acid from 3 per cent to concentrated and the length of hydrolysis from $\frac{1}{2}$ to $8\frac{1}{2}$ hours. In some cases Levene's (11) method of introducing a little stannous chloride into the hydrolyzing mixture was followed. It was soon discovered that the reducing power of the birds' nest was gradually diminished by heating in an acid solution. A difference of 6.37

TABLE II.

Relation between Quantity of Reducing Sugar and Time of Hydrolysis.

Time.	Reducing sugar.
hrs.	*per cent*
$\frac{1}{2}$	17.36
1	15.99
1	16.03
2	13.63
3	13.11
$8\frac{1}{2}$	10.99

per cent of carbohydrate calculated as glucose between the samples hydrolyzed $\frac{1}{2}$ and $8\frac{1}{2}$ hours is shown in Table II. In this experiment 20 per cent hydrochloric acid was used, the highest figure, in the table, 17.36 per cent, is much lower than that reported by Müller (9) for the carbohydrate in salivary mucin. His is 37 per cent, estimated by making phenylosazone from the hydrolyzed mixture.

Distribution of Nitrogen.

For the distribution of nitrogen Van Slyke's (12) method was closely followed, except that the bases were precipitated from a volume of 250 cc. instead of 200 cc. and the correction for solubility of the basic phosphotungstates made accordingly. Approximately 2 gm. of the ground and sifted birds' nest were taken for each of the four series of experiments. Several months elapsed between each one of the series.

By a study of Table III it will be seen that it was possible to obtain a fairly complete result on the distribution of nitrogen, the results for the different nitrogen fractions in the three finished series totalling 99.84, 100.61, and 100.54 per cent, respectively.

Series II and III are in close agreement with each other and Series IV slightly different, probably due to the fact that the material used in Series IV was purchased at a different time from the others. In Series II and III with the exception of cystine nitrogen, humin nitrogen, and the non-amino nitrogen of mono-amino-acids, the differences between duplicate series are all

TABLE III.

Distribution of Nitrogen in the Edible Birds' Nests.

Series	I		II		III		IV		Average of II and III.	
Nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Amide	1.07	10.15	1.03	10.06	1.05	10.10	1.09	10.54	1.04	10.08
Humin	0.71	6.75	0.65	6.38	0.72	6.98	0.70	6.77	0.69	6.68
Arginine	1.45	13.69	1.43	13.96	1.44	13.93	1.25	12.12	1.44	13.95
Cystine	0.37	3.51	0.29	2.79	0.41	4.00	0.49	4.77	0.35	3.39
Histidine			0.60	5.90	0.67	6.53	0.67	6.47	0.64	6.22
Lysine			0.26	2.55	0.25	2.37	0.19	1.84	0.26	2.46
Amino nitrogen of mono-amino-acids.			5.08	49.61	5.24	50.76	4.99	48.41	5.16	50.19
Non-amino nitrogen of monoamino-acids			0.87	8.54	0.61	5.90	1.00	9.62	0.74	7.22
Total nitrogen recovered			10.21	99.84	10.40	100.61	10.38	100.54	10.31	100.23
Total nitrogen determined.	10.57		10.24		10.33		10.31		10.29	

within the maximun experimental error allowed by Van Slyke (12) for pure proteins. The presence of considerable fine feathers with their high sulfur content and the resulting difficulty in obtaining a pure sample may be the cause of the variation in the cystine nitrogen. The differences in the humin nitrogen are probably due to the carbohydrate in the nest proteins.

The humin nitrogen is shown much higher than that of any of the pure proteins. The highest Van Slyke (12) has reported is 3.6 per cent for ox hemoglobin, a difference of 3.08 per cent from that of the nest. This high humin nitrogen may be explained as the result of the presence of a carbohydrate radicle in the nest proteins. Gortner and Blish (13), Gortner (14), and Hart and Sure (15) reported that the presence of dextrose or any other carbohydrate caused an increase of humin nitrogen during the hydrolysis of zein, fibrin, and casein.

The influence of the presence of a carbohydrate radicle on the distribution of nitrogen is also shown by the comparatively low lysine nitrogen and the high histidine nitrogen of the nest proteins. Thus, in case of pure casein reported by Hart and Sure (15) the value given for lysine nitrogen is 9.41 per cent and that for histidine nitrogen is 5.95 per cent. On the addition of dextrose, sucrose, and starch, respectively, during the hydrolysis of casein the corresponding values for lysine nitrogen are 7.01, 6.38, and 5.54 per cent, and those for histidine nitrogen are 7.31, 7.65, and 7.30 per cent. In every case, therefore, there is a decrease in the lysine nitrogen and an increase in the histidine nitrogen. Although the nature of the carbohydrate radicle in the nest has not yet been completely determined, it seems to possess in common with other carbohydrates the power of causing a redistribution of amino-acids on hydrolysis of protein. The presence of feathers in the material is undoubtedly the cause of the high value, 3.39 per cent for the cystine nitrogen in the nest. The highest value Van Slyke gave for cystine nitrogen in pure proteins is 1.25 per cent in gliadin.

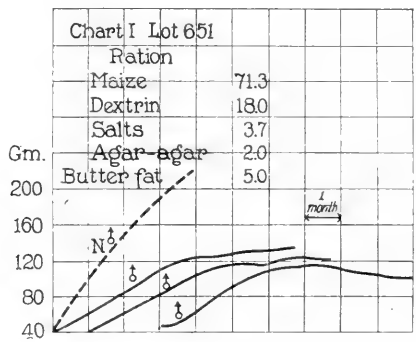
Biological Value of the Birds' Nests Proteins.

Feeding experiments were conducted on rats at Dr. McCollum's laboratory,[1] the School of Hygiene and Public Health, Johns Hopkins University, and the birds' nest was used to supplement a ration adequate in all respects but the character of the protein. Two unsatisfactory proteins, maize kernel and rolled oats, were chosen because they are of different character (16). Although

[1] For this part of the work I am indebted to Dr. McCollum and Miss Simmonds who kindly continued the experiments that I had started, and thus enabled me to secure the results.

when each is fed as the sole source of protein, they have approximately the same biological values for the support of growth, they are not at all similarly constituted. Oat proteins are supplemented well by the amino-acid mixture which comes from the digestion of gelatin, whereas the proteins of the maize kernel are not so supplemented in a degree which can be demonstrated by growth experiments with young animals.

The complexes which form the limiting factor in those two proteins are different, and, therefore, if the birds' nest protein has a high nutritive value it should at least supplement one of them. Since its addition failed to supplement either protein, it seems very probable that the birds' nest protein is of an inferior quality. It is of course possible that when taken together



with certain other foods it might have value, but its value is problematical in any case, and it is certain that it is far from being a complete food protein.

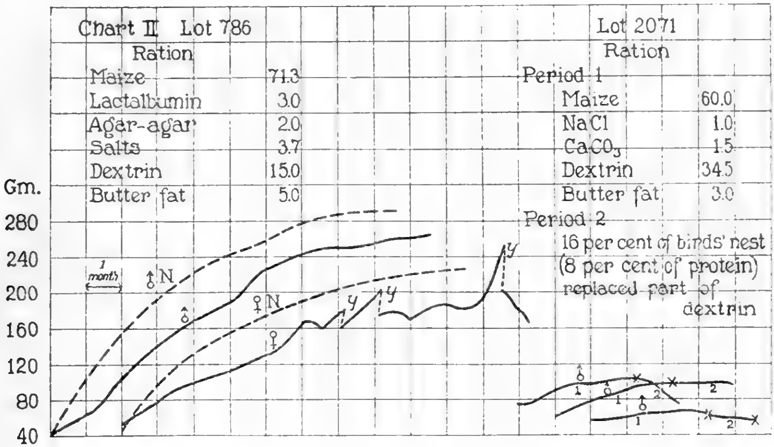
Lot 651 (Chart I) represents the growth curves for a group of rats which were restricted to a diet containing about 7 per cent of corn protein. The diet was otherwise fairly satisfactorily constituted. It failed to induce growth at a rate corresponding to about half the normal rate, and the animals became stunted when they were about half the normal adult size.

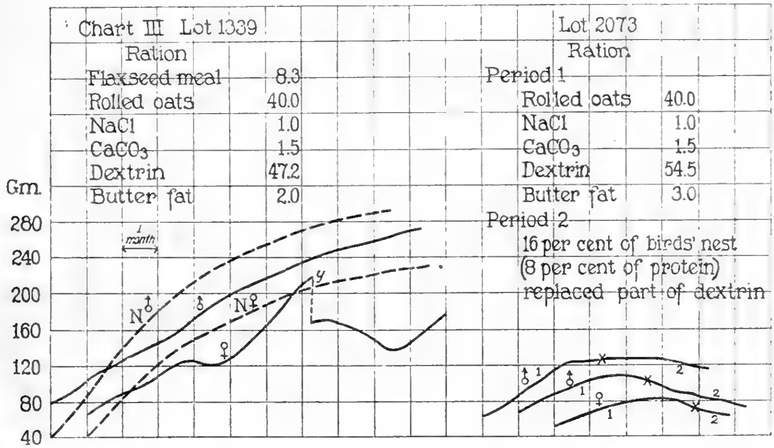
This diet is greatly improved by the addition of even so small an amount of purified protein as 3 per cent of lactalbumin. This is shown in the growth curves of Lot 786 (Chart II).

Lot 2071 (Chart II) shows the very slow rate of growth of a group of rats which were restricted to a diet comparable in all respects to those just mentioned except that the corn protein was fed at the plane of 6 per cent of the food mixture. This ration, when supplemented with a protein

having an appreciable biological value, would become capable of inducing growth, as illustrated in the case of Lot 786.

When a group of young rats were fed this diet for a period of 13 weeks and had been able to increase in weight but very little, and had finally completely failed to grow, 16 per cent of the Chinese edible birds' nest,





equivalent to 6 per cent of N × 6.25, was added in the place of a portion of the carbohydrate of the diet. If the birds' nests protein had any appreciable nutritive value, it should have enabled the young animals to respond with growth. A wide experience has demonstrated that such response will be observed when the protein added is of such a nature as to

furnish certain essential amino-acids which form the limiting factor in the proteins of the corn (maize). In this experiment there was no growth following this addition of birds' nests. The only conclusion which can be drawn is that the birds' nests do not supplement the protein and the corn kernel.

Lot 2073 (Chart III) shows that young rats cannot grow much when the diet contains only the amount of protein which will be furnished by a content of rolled oats equivalent to 40 per cent of the food mixture. This amounts to approximately 6 per cent of protein in the diet. Lot 1339 (Chart III) shows that the same food mixture, supplemented with the proteins of flaxseed oil meal sufficient to furnish an additional 3 per cent of proteins, is sufficient for the maintenance of a rate of growth somewhat more rapid than half the rate at which the young rat is capable of growing. Animals on this diet have reached approximately the full adult size, and young have been produced.

The fact that Lot 2073 in Period 2 failed to respond with growth when 16 per cent of the edible birds' nest was added to the diet, leaves no room for doubt that this substance, although a protein of a peculiar character, does not supplement the proteins of the oat kernel in any appreciable degree.

SUMMARY.

1. The Chinese edible birds' nest has the properties of a protein as well as those of a carbohydrate. It belongs, therefore, to the class of glycoprotein.

2. Its percentage composition resembles that of salivary mucin. Its ash is high, but there is no sandy material present. It contains 10.29 per cent nitrogen and at least 17.36 per cent carbohydrate.

3. Artificial digestion experiments indicated that the birds' nest was digested by both pepsin hydrochloric acid and trypsin at a slower speed than boiled egg.

4. The distribution of nitrogen showed a higher value for both humin nitrogen and cystine nitrogen than for pure proteins. The former is probably due to the carbohydrate radicle in the nest while the latter is due to the presence of fine feathers. Other fractions were similar to those of pure proteins.

5. Feeding experiments indicate that the nest protein is probably of an inferior quality. It failed to supplement a ration adequate in all respects, except that the source of protein was derived from either maize kernel or rolled oats. Although both of them were unsatisfactory proteins they were different in character.

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THE ISOLATION AND THE NATURE OF THE AMINO SUGAR OF CHINESE EDIBLE BIRDS' NESTS.*

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Literature on Carbohydrate Radicle of Glycoproteins.

Although many attempts have been made by different investigators to isolate the carbohydrate group from various glycoproteins, only two amino sugars have so far been isolated: chitosamine (glucosamine) and chondrosamine. Chitosamine (1) was first isolated from lobster shells and later from other substances. In some cases the hydrochloride of chitosamine was obtained in crystalline form while in others the base was estimated either from the reducing power of the hydrolysis solution or from the phenylosazone prepared from it. Bywaters (2) estimated the percentage of chitosamine in the white of eggs by the former method, and Osborne, Jones, and Leavenworth (3) by the latter. According to the latter authors the reduction method for the estimation of chitosamine is of but little value. Ross (4) succeeded in isolating the glycosamine hydrochloride in crystalline form from *Boletus edulis*. Her method is similar to the standard one given by Fischer (5) using concentrated hydrochloric acid to hydrolyze the substance. She demonstrated the fact that the glucosamine could not be isolated by hydrolyzing the substance with sulfuric acid. Ostwald (6) isolated a small amount of chitosamine from ovomucoid by dialyzing the hydrolysis solution with distilled water and evaporating the dialysate. Müller (7) made an investigation on the carbohydrate radicle of mucin from the sputum of tuberculosis patients and from the submaxillary saliva of dogs, and after having encountered many failures he succeeded in isolating a crystalline compound he called glucosamine hydrochloride. The properties of this chitosamine hydrochloride are somewhat different from those summarized by Levene and La Forge (8) for the ordinary chitosamine hydrochloride, in that Müller's is soluble in 95 per cent alcohol, while the latter is almost insoluble in 80 per cent

* The work reported in this article was conducted at the Nutrition Laboratory, Department of Home Economics, University of Chicago. It forms part of the thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Chicago.

alcohol; and that his is destroyed by standing in hydrochloric acid solution and therefore the work has to be carried through rapidly, while the latter is ordinarily prepared by hydrolyzing the substance with concentrated hydrochloric acid for many hours and then evaporating it on a water bath.

Levene (8, 9, 10) has made a most extensive study of the carbohydrate radicle of mucin from pigs' stomach, funis mucin, humor vitreous mucoid, cornea mucoid, and mucoids from cartilage and tendon. From the mucins he isolated the chitosamine while from the mucoids the chondrosamine. The difference between the two will be discussed later.

Isolation of the Carbohydrate.

In attempting to isolate the carbohydrate of the nest, the methods of the previous investigators were first tried, but met with only very moderate success or entire failure. However, since the experiments showed something of the properties of the carbohydrate in contrast with the properties of the previously known amino sugars, they are briefly described here.

Fischer's (5) method of preparing chitosamine by hydrolyzing the substance with concentrated hydrochloric acid and then evaporating it on a water bath was first tried. This method met with entire failure, as the reducing power of the birds' nest which had been so strong in a dilute hydrochloric acid solution hydrolyzed for a short time, was completely destroyed.

Levene's (11) method was next followed, isolating chitosamine from mucins by preparing the conjugated sulfuric acid first and then hydrolyzing the product with 20 per cent hydrochloric acid and a little stannous chloride. In following this method occasionally a few crystals of various forms, among which "Kreissector" described by Müller (7) was also present, were obtained; but in no case was the yield sufficient for any analytical study.

The third method used was Müller's (7) for preparing chitosamine from salivary mucin. About 15 gm. of the nest were hydrolyzed with 150 cc. of 4 per cent hydrochloric acid for 3 hours on a water bath. The hydrolysis mixture was treated at once with dialyzed iron which threw down a heavy brownish black precipitate. The dark brown filtrate had a strong reducing power, but after evaporating in vacuum desiccators only a very few oblong crystals were formed which were not enough for recrystallization.

During the many failures some of the characteristic properties of the carbohydrate of the birds' nest were observed. It is unstable toward alkali and acid plus heat. It is soluble in 95 per cent ethyl and methyl alcohol. It seems to be partially absorbed or destroyed by protein precipitants. It is, therefore, essential to hydrolyze the material with dilute hydrochloric acid only long enough to split off the carbohydrate radicle but not to destroy it. The following method of preparation was then applied and 10 gm. of beautiful white crystals were obtained after three crystallizations from alcohol and ether.

About 300 gm. of the birds' nest were heated with 2,100 cc. of 3 per cent hydrochloric acid for 5 hours until the material had completely gone into solution, but no black precipitate was produced. (On long heating a black precipitate results.) The hydrolysis solution was evaporated to dryness over sulfuric acid and under solid sodium hydroxide in vacuum desiccators at room temperature. The thick black residue was extracted with 95 per cent alcohol and the alcoholic solution was separated by centrifuge. The extraction was continued until the alcoholic solution gave a little or no reducing power. It required from fifteen to twenty extractions, at least. The alcoholic solution was evaporated to a very thick dark brown syrup under diminished pressure (30 mm.). In another case where a better yield was obtained it stood for several months and evaporated gradually at room temperature. The syrup was then taken up with a large quantity (about 500 cc.) of methyl alcohol. A fairly large quantity of brownish crystals thus resulted. They were filtered off and redissolved in a little boiling water. The solution was filtered again and the brownish filtrate was treated with about eight times its volume of absolute alcohol and then with ether until no more precipitate formed. The crystallization was repeated until the crystals became pure white and seemed to be uniform on microscopic appearance; rods grouping themselves in the forms of an elaborate fern leaf. The yield was about 10 gm.

As the properties of this product seemed to be more or less different from those of any of the known amino sugars, it was thought possible that the product might be a mixture instead of being a single compound. Another preparation was, therefore, made according to the method given above except that in dissolving the brown crystals from methyl alcohol less water was used and instead of using both absolute alcohol and ether to bring about the precipitation, only the former was used. This required much more alcohol, at least 1,500 cc. of absolute alcohol. The alcoholic solution which was only cloudy at first, yielded a large

crop of about 5 gm. of beautiful precipitate on standing over night. It was filtered by suction and after being recrystallized twice, was dried over sulfuric acid. There are various forms of crystals in this fraction, among which the "Kreissector" form of Müller predominates.

The alcoholic filtrate was treated with ether until the solution became cloudy. On standing over night another crop of about 5 gm. of pure white crystals was obtained. It was filtered and dried as before. The crystals of this fraction are large fluffy flakes.

Although the largest yield of the purified amino sugar was only about 3 per cent, the original protein contained at least 17.36 per cent of reducing substance as shown in the previous report. The small yield might be due to either the unstability of the amino sugar or the presence of some reducing substance other than amino sugar in the protein. Judging from the properties of the sugar, the former hypothesis is probably correct.

Properties of the Three Sets of Crystals.

The three sets of crystals are all very soluble in water and 80 per cent alcohol, fairly soluble in 95 per cent and in methyl alcohol, slightly soluble in absolute alcohol, and insoluble in ether, chloroform, and acetone. They all taste sweet and give a strong test with both Molisch's and Fehling's reagents. They fail to respond to any of the protein reactions. When boiled with strong sodium hydroxide, they give off ammonia. None of them melted when they were placed side by side in melting point tubes and heated to 250°C. in a glycerol bath. However, the alcohol-ether crystals started to turn dark at 142°C., the alcohol fraction at 170°C., and the ether fraction at 150°C. At 250°C. they all became almost black in color. On analysis they give the following percentage composition:

I. Analytical data of the alcohol-ether crystals.
   A. Determination of carbon and hydrogen.
      1. 0.1858 gm. substance: 0.2254 gm. carbon dioxide and 0.1121 gm. water.
      2. 0.1631 gm. substance: 0.1978 gm. carbon dioxide and 0.0980 gm. water.

B. Determination of nitrogen by combustion.

1. 0.1860 gm. substance: 10.1 cc. nitrogen at 20°C. and 758 mm. pressure.
2. 0.1801 gm. substance: 10.1 cc. nitrogen at 20.5°C. and 756 mm. pressure.

C. Determination of chlorine.

1. 0.1541 gm. substance required for titration of hydrochloric acid 13.92 cc. 0.05 N silver nitrate.
2. 0.1597 gm. substance required for titration of hydrochloric acid 14.35 cc. 0.05 N silver nitrate.
3. 0.1594 gm. substance required for titration of hydrochloric acid 14.41 cc. 0.05 N silver nitrate.

$C_6H_{13}O_5NCl$. Calculated. H 6.50, C 33.41, N 6.51, Cl 16.45.
Found. H 6.69, C 33.09, N 6.28, Cl 15.99.

D. Determination of the optical activity.

1. 0.3335 gm. substance in 9.947 gm. water rotated in a 1 dm. tube with D-light +2.58° 7 minutes after the solution was made, and +2.38° 28 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{20} = +79.5°$	$[\alpha]_D^{20} = +73.4°$

2. 0.2821 gm. substance in 10.816 gm. water rotated in a 1 dm. tube with D-light +1.81° 10 minutes after the solution was made, and +1.72° 20 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{20} = +71.2°$	$[\alpha]_D^{20} = +67.7°$

Average of (1) and (2).

Initial.	Equilibrium.
$[\alpha]_D^{20} = +75.4°$	$[\alpha]_D^{20} = +70.6°$

II. Analytical data of the alcohol fraction.

A. Determination of nitrogen by Kjeldahl-Gunning method.

1. 0.3140 gm. substance required for titration of ammonia 14.56 cc. 0.1 N sulfuric acid.
2. 0.2947 gm. substance required for titration of ammonia 13.42 cc. 0.1 N sulfuric acid.
3. 0.1199 gm. substance required for titration of ammonia 5.54 cc. 0.1 N sulfuric acid.

$C_6H_{13}O_5NCl$. Calculated. N 6.51.
Found. N 6.46.

B. Determination of optical activity.

1. 0.3056 gm. substance in 9.9028 gm. water rotated in a 1 dm. tube with D-light +2.703° 10 minutes after the solution was made, and +2.153° 22 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +87.6°$	$[\alpha]_D^{10} = +69.7°$

2. 0.2997 gm. substance in 10.0797 gm. water rotated in a 1 dm. tube with D-light +2.56° 15 minutes after the solution was made, and +2.12° 20 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +86.1°$	$[\alpha]_D^{10} = +71.3°$

Average of (1) and (2).

Initial.	Equilibrium.
$[\alpha]_D^{10} = +86.9°$	$[\alpha]_D^{10} = +70.5°$

III. Analytical data of the ether fraction.

A. Determination of nitrogen by Kjeldahl-Gunning method.

1. 0.2152 gm. substance required for titration of ammonia 9.68 cc. 0.1 N sulfuric acid.
2. 0.2016 gm. substance required for titration of ammonia 9.02 cc. 0.1 N sulfuric acid.

$C_6H_{13}O_5NCl$. Calculated. N 6.51.
Found. N 6.29.

B. Determination of optical activity.

1. 0.2329 gm. substance in 9.8419 gm. water rotated in a 1 dm. tube with D-light +1.596° 10 minutes after the solution was made, and +1.675° 22 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +67.5°$	$[\alpha]_D^{10} = +70.8°$

2. 0.3512 gm. substance in 10.2822 gm. water rotated in a 1 dm. tube with D-light +2.24° 10 minutes after the solution was made, and +2.425° 20 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +65.6°$	$[\alpha]_D^{10} = +71.0°$

Average of (1) and (2).

Initial.	Equilibrium
$[\alpha]_D^{10} = +66.6°$	$[\alpha]_D^{10} = +70.9°$

The phenylosazone was prepared from the alcohol-ether crystals, Garard and Sherman's (12) method was followed. About 1 gm. of the substance was placed in a small beaker with 10.80 gm. of freshly distilled phenylhydrazine, 12.60 gm. of glacial acetic acid, and 6.85 gm. of pure sodium acetate. The volume was made up with distilled water to 100 cc. and the mixture heated on a water bath for 3 hours. Unlike the osazone from glucose which crystallizes out during the course of heating under the same conditions, the osazone from this substance remained entirely in solution until it was cooled over night. This is similar to a preparation from glucosamine. It was so soluble in the 60 per cent alcohol used by Garard and Sherman that to recrystallize their glucosazone that solvent could not be used and Levene's method of recrystallization from water and pyridine was followed. After two recrystallizations it gave a melting point of 214° with rapid heating. On standing over sulfuric acid the color darkens and the melting point lowers markedly—170° in one case during a week's standing. Samples of glucosazone made in exactly the same way for comparison both from glucose and glucosamine gave the usual melting point of 208°, and did not change on standing over sulfuric acid. Three tubes, one containing the

unknown osazone, the second glucosazone, and the third a mixture of the two, were heated in one bath. The unknown always melted at a temperature about 6° above the others, while the mixture was either about the same or a little below the glucosazone.

An attempt was made to study the optical activity of the osazone. As it did not dissolve completely in the usual amount of Neuberg's (13) alcohol pyridine mixture even after having stood for an hour with occasional shaking, the amount of solvent was doubled and a clear brown solution was secured after half an hour's standing. The color of the solution was, however, so deep that it was impossible to see through a 1 dm. tube. The solution was, therefore, diluted to one-fourth, the strength recommended by Neuberg and by Levene and it was found to rotate slightly to the left.

DISCUSSION.

An examination of the properties and the analytical data of the three sets shows that they are all hexosamine hydrochlorides and that they differ from each other only in their optical rotation and the temperature at which they begin to turn dark on heating. The latter difference might be explained on the basis of their purity. The one which is probably the purest, the alcohol fraction, turns dark at the highest temperature, 170°C., while the alcohol-ether crystals which begin to turn dark at 142°C., might be the least pure of the three. The degree of purity of the three sets of crystals is also shown by the slight difference in their nitrogen content. The alcohol fraction has a value of 6.46 per cent, the ether fraction 6.29 per cent, and the alcohol-ether crystals 6.28 per cent. The calculated value for the percentage of nitrogen of hexosamine is 6.51.

The difference in the optical rotation is, however, most interesting. The average of the two determinations of optical rotation of the alcohol-ether crystals is $[\alpha]_D^{20} = +75.4°$ to $+70.6°$ while those of the alcohol fraction and the ether fraction are $[\alpha]_D^{10} = +86.9°$ to $+70.5°$ and $[\alpha]_D^{10} = +66.6°$ to $+70.9°$, respectively. It is, therefore, clear that the three sets of crystals differ only in their initial rotations. The alcohol-ether crystals and the alcohol fraction have a descending rotation, while the ether fraction has an ascending one. Furthermore,

the value of the initial rotation of the alcohol-ether crystals lies between those of the other two fractions and is almost equal to the mean of the other two, which is + 76.8°. The duplicate determinations were made at least 6 months apart. These facts seem to indicate that the alcohol fraction is probably one of the hexosamine hydrochlorides of the α form; the ether fraction the β form, and the alcohol-ether crystals a mixture of the two. Thus Levene (14) reported that the optical rotation of chondrosamine hydrochloride of the α form was $[\alpha]^{\circ}_{D} = +\ 129.5°$ to $+\ 93.8°$ and that of the β form was $[\alpha]^{\circ}_{D} = +\ 62.7°$ to $+\ 91.1°$. No report on the properties of the α and β forms of other hexosamine hydrochlorides could be found in the literature.

Unfortunately, there is not enough material for more experimental work at present and the question as to whether the above hypothesis is correct has to be left unanswered.

What one of the possible hexosamines this is, has not been determined. A study of Tables I and II indicates that the properties of the unknown sugar are not in close agreement with either of the two known hexosamine hydrochlorides; the chitosamine hydrochloride or the chondrosamine hydrochloride. From Müller's (7) description of the "glucosamine" which he obtained from sputum mucin it seems probable that his substance was the same as the alcohol-ether crystals. The highest melting point which he gave from his osazone was only 205°, but the discrepancy with our 214° might be explained by the greater purity of the latter.

The unknown hexosamine hydrochloride differs from chondrosamine in its melting point and its optical activity, but resembles it in its solubility in alcohol and in giving an unstable osazone.

The most striking point of resemblance between chitosamine hydrochloride and the hydrochloride of the unknown sugar lies in their optical activity and their stability toward concentrated hydrochloric acid, solubility in alcohol, and in the stability and the character of their osazone. Thus, chitosamine hydrochloride is insoluble in 80 per cent alcohol, while the hydrochloride of the unknown sugar is very soluble in 80 per cent alcohol, and when pure, soluble in 95 per cent alcohol, methyl alcohol, as well as absolute alcohol chitosamine hydrochloride may be prepared by boiling with concentrated hydrochloric acid, while this sugar is

TABLE I.

Comparison of the Properties of Hexosamine Hydrochlorides.

	Chondrosamine.	Chitosamine (glucosamine).			Unknown sugar.		
					Author.		
Investigator.	Levene.	Levene.	Müller.	Ordinary.	Alcohol-ether crystals.	Alcohol fraction.	Ether fraction.
Solubility in alcohols.							
80 per cent	Soluble.			Insoluble.	Very soluble.	Very soluble.	Very soluble.
95 per cent			Soluble.	Insoluble.	Soluble.	Soluble.	Soluble.
Absolute					Slightly soluble.	Slightly soluble.	Slightly soluble.
Methyl	Soluble.	Insoluble.			Soluble.	Soluble.	Soluble.
Stability in concentrated HCl			Unstable.	Stable.	Unstable.	Unstable.	Unstable.
Melting point	182°C.	No.	No.	No.	No.	No.	No.
Temperature at which sugar turns dark					142°C.	170°C.	150°C.

TABLE II.

Comparison of Optical Rotation of Hexosamine Hydrochloride.

Chondrosamine.		Chitosamine (glucosamine).			Unknown sugar.	
Levene.		Levene.		Ordinary.	Author.	
Origin.	$[\alpha]_D$	Origin.	$[\alpha]_D$	$[\alpha]_D$	Precipitation.	$[\alpha]_D$
	α form.	Funis mucin.	+84.8° to +71.9°	+100° to +73°	Alcohol-ether.	+75.4° to +70.6°
Tendon.	+129° to +93°	Stomach mucin.	+90.1° to +70.6°		Alcohol.	+86.9° to +70.5°
	β form.	Vitreous humor.	+96.8° to +72.2°		Ether.	+66.6° to +70.9°
Synthetic.	+60° to +91°	Cornea mucoid.	+95.4° to +71.9°			
Aorta mucoid.	+65.6° to +91.2°	Serum mucoid.	+89.5° to +71.4°			
Sclera mucoid.	+56.8° to +95.7°					

completely destroyed. Glucosazone melts at 208° and the osazone from this sugar at 214°. The former is more soluble in Neuberg's alcohol-pyridine mixture. It is stable on standing over sulfuric acid. The unknown osazone is unstable, darkening in color and lowering its melting point.

Which amino-hexose this sugar is is therefore still undetermined.

Levene (10) has recently made a grouping of his glycoproteins and their derivatives which throws light on the question of the birds' nest glycoproteins from tendons, cartilage, aorta, and sclera mucoids. He has isolated the conjugated sulfuric acid which he calls chondroitin sulfuric acid and which gives chondrosamine on hydrolysis, and from gastric mucosa, vitreous humor, funis mucin, cornea mucoids, and serum mucoids he has isolated mucoitin sulfuric acid from which chitosamine (glucosamine) is derived. Chondroitin sulfuric acid is highly resistant to hydrolytic agents, and mucoitin sulfuric acid is very easily hydrolyzed, and even amino sugar is often destroyed completely by the hydrolytic agent. It is plain from the work described in this paper that birds' nest glycoprotein belongs to the second group.

He also found that different members of the second group behave differently, especially in the properties of their conjugated sulfuric acid so that he distinguishes two subdivisions A and B. The precursor of the unknown sugar of birds' nest is more like subgroup B than A. Levene's subgroup A gives a fairly insoluble barium salt of the conjugated sulfuric acid and the subgroup B (the description of which was published after the work in this paper was done) a distinctly soluble one. The efforts of the writer to isolate a conjugated sulfuric acid from the birds' nest by the Levene early method met with almost complete failure, the substance behaving in the manner later described by Levene for subgroup B. It is, therefore, certain that the precursor of the unknown sugar belongs to the subgroup B of the second group of glycoproteins described by Levene.

SUMMARY.

1. A method for the solution of the amino sugar from Chinese edible birds' nest is described.

2. Three sets of pure white crystals having the percentage composition of a hexosamine, were obtained. They are similar

in all their properties except optical rotation which for two of them decreases on standing over night and of the third increases. The probable conclusion is that one of the two sets showing a decreasing of the optical rotation on standing is the α form of a hexosamine, the one which has a rise of the rotation the β form, and the third set a mixture of the two.

3. Levene's recent description of the different groups of glycoproteins and their derivatives shows that there is a striking resemblance between the properties of the precursor of this unknown sugar and those of his subgroup B of the second group.

In conclusion I wish to express my obligation to Dr. Katharine Blunt, in whose laboratory the work reported in the last two papers was carried out, for her helpful suggestions and constant interest in the investigation.

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ANIMAL CALORIMETRY.

Eighteenth Paper.

THE BEHAVIOR OF VARIOUS INTERMEDIARY METABOLITES UPON THE HEAT PRODUCTION.*

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CONTENTS.

I. INTRODUCTION.

The behavior of the ordinary foodstuffs during successive hours after their ingestion as they react in the organism of the dog has been the subject of numerous former researches published from this laboratory. The last paper upon this theme was issued in 1919 (1) and dealt with the similarity of the extent of the rise in heat production after giving lactic acid and alanine. It was then

* An abstract of this paper was presented before the Physiological Congress held in Paris, July, 1920.

(erroneously) concluded that the ingestion of the two substances resulted in the same physiological reaction upon metabolism.

In 1912 the writer (2) proved that glycine and alanine were powerful stimuli to increased heat production. At the same time Benedict (3) advanced the theory that the specific dynamic action of the foodstuffs was due to stimuli arising from the formation of organic acids. This has been elsewhere discussed by me (4).

In the light of our present knowledge it seemed desirable to investigate the comparative behavior of various simple substances, such as acetic, glycollic, lactic, and hydrochloric acids; the behavior of glycine after neutralization with sodium bicarbonate, of sodium lactate and sodium glycollate, and of sodium bicarbonate itself. In this manner one might discover to what extent the acidic radicle was a dominant factor influencing metabolism. Since strong solutions of acetic acid were vomited by the dog, one could administer only small quantities to the animal. As noted in the previous paper, it was found possible to administer materials like lactic acid in a solution containing 2.5 gm. of Liebig's extract of beef warmed to a temperature of 38°C. For the reason of the presence of Liebig's extract in the diet, the protein metabolism throughout the whole series of experiments was estimated on the basis of a urinary nitrogen elimination of 0.132 gm. per hour, as was determined for the basal metabolism of the dog employed. The figures given represent in general the measurements of the metabolism obtained during the 2nd and 3rd hours after administering the substances. The results obtained in this paper should be considered in the light of the paper by Miss Taistra (5) which follows this.

II. THE BASAL METABOLISM.

A dog was given the "standard diet" (6) at 5 p.m. the day before the experiment and also regularly throughout the entire period of experimentation. The basal metabolism was determined about 18 hours after the ingestion of this maintenance diet. The results are summarized in Table I.

According to these determinations the basal metabolism by indirect calorimetry averages **17.61** calories per hour and the respiratory quotient **0.80.**

TABLE I.

Basal Metabolism of Dog XIX.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
6	Jan. 15	2	0.82	35.59	37.54	+1.95
19	Feb. 5	2	0.84	33.12	33.52	+0.40
24	Mar. 16	2	0.80	35.37	31.30	−4.05
29	" 30	2	0.76	36.59	36.14	−0.45
32	Apr. 6	2	0.75	36.52	35.78	−0.74
35	" 12	2	0.79	34.75	34.80	+0.05
38	" 21	1	0.81	16.90	16.75	−0.15
Total..		13	0.80	228.84	225.83	−3.01 = 1.3 per cent

III. LIEBIG'S EXTRACT OF BEEF.

The influence of 2.5 gm. of this material, when given with 500 cc. of water to Dog XVIII, was found to increase the basal metabolism by 1.1 and 0.8 calories per hour, as has already been described by Atkinson and Lusk (1). In the present instance the administration of 2.5 gm. of the substance in a smaller quantity of water, 400 cc., caused a scarcely appreciable rise in heat production above the basal level. The experiments may be thus expressed:

TABLE II.

Metabolism of Dog XIX after Administering a Broth Containing 2.5 Gm. of Liebig's Extract of Beef.

Experiment No.	Date.	Volume of the broth.	Duration of experiment.	R. Q.	Calories per hour.		
					Indirect.	Direct.	Difference.
	1920	*cc.*	*hrs.*				
7	Jan. 16	350	2	0.79	36.53	34.38	−2.15
14	" 28	400	2	0.81	38.93	43.41	+4.48
21	Feb. 9	400	1	0.82	19.79	19.99	+0.20
23	" 19	400	2	0.81	34.25	37.10	+2.85
25	Mar. 19	400	2	0.80	34.72	34.06	−0.66
34	Apr. 9	400	2	0.82	33.91	33.22	−0.69
Total..			11	0.81	198.13	202.16	+4.03 = 2.0 per cent

The average heat production, as determined by indirect calorimetry after giving 400 cc. of a broth containing 2.5 gm. of meat extract, was 18.1 calories per hour, or 0.49 calory above the basal metabolism. The difference is so slight as to be negligible. The average respiratory quotient 0.81 is essentially the same as that of the basal metabolism.

IV. SODIUM BICARBONATE.

In order to detect the influence of sodium bicarbonate, 8 gm. of the material were ingested with 300 cc. of water and 2.5 gm. of Liebig's extract, with the following experimental results:

TABLE III.

Dog XIX after Administering a Broth Containing 8 Gm. of $NaHCO_3$, 2.5 Gm. of Liebig's Extract, and 300 Cc. of Water.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories per hour.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
42	May 1	2	0.84	37.22	37.58	+0.36
43	" 4	1	0.82	16.95	17.72	+0.77
Total....		3	0.83	54.17	55.30	+1.13 = 2.1 per cent

The rate of metabolism averages here 18.05 calories per hour, an increase of 0.45 above the basal metabolism, about the same as when the broth alone was given. The increase may be considered negligible.

V. ACETIC ACID.

The behavior of acetic acid is of especial interest in that it is probably a cleavage product of fatty acid as a consequence of its β-oxidation. Acetic acid on combustion manifests a respiratory quotient of 1.00 and 1 gm. of the substance requires 1.067 gm. of oxygen and yields 3.491 calories of heat. One may calculate that 1 liter of oxygen thus employed would correspond to the formation of 4.674 calories. This is essentially the same value for oxygen as when a liter of it oxidizes fat and produces 4.686 calories with the respiratory quotient 0.71.

The following results were obtained in the dog after ingesting 3 gm. of acetic acid:

TABLE IV.

Dog XIX after Administering 3 Gm. of Acetic Acid in Broth Containing 2.5 Gm. of Liebig's Extract of Beef and 400 Cc. of Water.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories per hour.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
4	Jan. 12	2	0.76	42.55	42.52	−0.03
12	" 23	2	0.79	41.25	42.77	+1.52
13	" 27	2	0.78	40.63	38.33	−2.30
Total...		6	0.78	124.43	123.62	−0.81 = 0.6 per cent

This indicates that the metabolism of the dog, following the ingestion of 3 gm. of acetic acid containing 10.5 calories, rises to an average of 20.74 calories per hour, an increase of 3.13 calories above the basal level.

An unusual phase of this work is the presence of a low respiratory quotient following the ingestion of a material having a high quotient. Curiously enough, the same phenomenon of a reduced respiratory quotient appeared also when glucose and acetic acid were given together.

The experiments are all calculated without reference to the special modifications induced through the combustion of acetic acid. If, however, one recalculates the 1st hour of Experiment 12, in which the respiratory quotient was 0.83, on the hypothesis that as large a quantity as 1 gm. of acetic acid was oxidized during that interval, then the non-protein respiratory quotient for fat plus carbohydrate would reach the artificial value of 0.68. On this basis the calories of metabolism would be derived as follows: protein 3.50, acetic acid 3.49, fat 12.72, total 19.71. This contrasts with a total of 20.30 calories determined by the usual mode of calculation, a diminution of 0.59 calory for the hour. The usual method of calculating the results cannot therefore be wide of the mark.

It is possible that acetic acid may in part have been reduced in the organism to ethyl alcohol, which would explain the low respiratory quotient obtained, but of this there is no actual evidence. The experimental evidence shows that administration of acetic acid does not reduce the carbon dioxide-combining power

of the blood, hence one may conclude that it is rapidly absorbed and oxidized (5).

In a former experiment (Experiment 30, Dog III (7)) the administration of 5.8 gm. of ethyl alcohol, which if oxidized to acetic acid would have given rise to 7.6 gm. of the latter substance, effected an increase in the basal metabolism of only 0.9 calory per hour. It is well known that alcohol is only slowly oxidized, whereas acetic acid appears to be rapidly consumed.

VI. LACTIC ACID.

Lactic acid is believed by many to be a normal intermediary metabolite of glucose (8-12) as well as of alanine. Its behavior in metabolism may therefore merit further investigation. Solutions of 4.8 gm. were given to Dog XIX with the following results:

TABLE V.

Dog XIX after Administering 4.8 Gm. of Lactic Acid in Broth Containing 2.5 Gm. of Liebig's Extract and 400 Cc. of Water.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories per hour.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
10	Jan. 21	2	0.83	39.91	38.70	−1.21
11	" 22	2	0.84	41.14	40.34	−0.80
Total....		4	0.84	81.05	79.04	−2.01 = 2.5 per cent

Lactic acid, 4.8 gm., brought the rate of heat production to 20.26 calories per hour, an increase above the basal metabolism of 2.65 calories. The respiratory quotient of 0.84 is slightly higher than the average basal value of 0.80, in conformity with the fact that the respiratory quotient of lactic acid is 1.00.

The administration of lactic acid in this quantity temporarily reduced the CO_2-combining power of the plasma of this dog (5) and yet the increase in the heat production was less than was observed in the case of acetic acid administration where no reduction was observed.

To determine whether the administration of lactic acid in the form of an alkaline salt would abolish its specific dynamic influence, 10 gm. of sodium lactate were given, with the following results:

TABLE VI.

Dog XIX after Giving 10 Gm. of Sodium Lactate in Broth Containing 2.5 Gm. of Liebig's Extract and 400 Cc. of Water.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories per hour.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
33	Apr. 8	2	0.82	38.11	36.29	−1.82
36	" 13	2	0.81	37.99	36.42	−1.57
Total...		4	0.82	76.10	72.71	−3.39 = 4.5 per cent

By the indirect method the heat production is 19.02 calories per hour, an increase of 1.41 calories above the basal. The respiratory quotient is that usually found. The urine eliminated at the end of the experiments was weakly alkaline to litmus.

If the sodium lactate were oxidized to $NaHCO_3$, the chemical reaction would be $C_3H_5O_3Na + 3O_2 = NaHCO_3 + 2CO_2 + 2H_2O$. Under these circumstances the respiratory quotient would be 0.667 and the quantity of oxygen absorbed the same as would be required to oxidize lactic acid itself. Since the respiratory quotient actually found is 0.82, it is evident that the alkali could not have been entirely, or even largely, neutralized by carbonic acid.

The increase of 1.4 calories caused by 8 gm. of lactic acid administered as a sodium salt is not as great as that of 2.7 calories when only 4.6 gm. were given in the form of the free acid. This might be due to the fact that the presence of alkali promotes the conversion of lactic acid into glycogen. It should be recalled that the ingestion by a dog of 8 gm. of glucose into which 8 gm. of lactic acid are convertible has no effect whatever upon the metabolism (1). The intermediary metabolite is therefore more immediately available for use by the cell than is glucose which may be at once dehydrated and put aside as glycogen. To some this may appear to favor the theory that glucose must become glycogen before it can be utilized.

VII. GLYCOLLIC ACID.

Glycollic acid has been suggested as the primary product following the deamination of glycine. As such it might be the cause of the profound stimulation of metabolism which follows the administration of glycine itself. The literature concerning the intermediary metabolism of glycollic acid is given by Sieburg and Vietense (13). Pohl (14) could find neither glycollic nor oxalic acid in the urine of a dog to which he had given the substance. Mayer (15), however, gave 10 gm. of glycol to a rabbit and recovered one-sixth of it in the form of glycollic acid in the animal's urine. Dakin (16) noted a rise in urinary ammonia from 0.12 to 0.22 gm. per day after giving 1.1 gm. of glycollic acid to a dog weighing 11 kilos.

In combustion glycollic acid manifests a respiratory quotient of 1.33. When 1 gm. is oxidized 0.632 gm. of oxygen is consumed and 2.206 calories are liberated. Each liter of oxygen employed has a value of 4.990 calories or approximately the same value as when glucose is oxidized.

Glycollic acid, 7.6 gm., containing about twice the number of potential H ions present in the quantities of acetic and lactic acids previously administered, were given to the dog and the results were as follows:

TABLE VII.

Dog XIX after Giving 7.6 Gm. of Glycollic Acid in Broth Containing 2.5 Gm. of Liebig's Extract and 400 Cc. of Water.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
20	Feb. 6	2	0.93	39.01	31.51	−7.50
22	" 18	2	0.96	37.33	36.01	−1.32
26	Mar. 20	2	0.91	38.27	34.14	−4.13
Total..		6	0.94	114.61	101.66	−12.95
37*	Apr. 20	3	0.88	55.47	50.91	−4.56

* Material given as a sodium salt.

The metabolism calculated by the indirect method is 19.10 calories per hour or an increase of 1.49 calories above the basal.

The high average respiratory quotient of 0.94 betokens the oxidation of the ingested material.

Administration of glycollic acid produced a profound fall in the CO_2-combining power of the blood plasma (5), indicating that a considerable quantity of the salt combines with the alkali of the blood and circulates in it.

This reduction in CO_2-combining power from 60 to 40 volumes per cent might have caused the elimination of 100 cc. of CO_2 from 500 cc. of blood presumably contained in the dog of 10 kilos body weight. Even though the whole of this elimination should have fallen upon the 2nd and 3rd hours recorded in the above experiments the increase in the respiratory quotient would have been only 0.02. Bicarbonate withdrawn from the tissues into the blood may have materially increased the CO_2 output.

The great reduction in the CO_2-combining power of the blood, with only a slight increase in the total metabolism, indicates that acid metabolites, when ingested, do not necessarily stimulate metabolism because of their acid properties.

When glycollate of sodium was given to the dog the increase above the basal metabolism was 0.88 calory per hour. The respiratory quotient was 0.88, indicating the combustion of the substance. If the salt had been oxidized with the production of $NaHCO_3$, its respiratory quotient would have been 0.80. The quotient obtained indicates that factors other than CO_2 for the neutralization of the alkali were brought into play.

An interesting phenomenon in relation to the ingestion of both glycollic acid and its salt is the reduced measured heat output of the dog despite the slightly increased heat production as computed from the oxidation process. This was also noticed when hydrochloric acid was ingested. It is evident, therefore, that heat was stored in the body which was not reflected by the rectal thermometer carried by the animal. There might have been an altered distribution of the blood supply so that some portion of the body attained an increased temperature. These experiments confirm the idea that for short periods the indirect method of heat production is usually more to be relied upon than the direct measurement.

VIII. HYDROCHLORIC ACID.

Atkinson and Lusk (17) demonstrated that the administration of 200 cc. of 0.4 per cent hydrochloric acid will increase the hourly heat production in the dog from 20 to 21.25 calories per hour, an increase of 1.25 calories.

In Experiment 28, 300 cc. of water containing 0.6 per cent of hydrochloric acid and 2.5 gm. of Liebig's extract were given to Dog XIX. The heat production as calculated was 19.16 calories per hour, an increase of 1.55 calories above the basal.

The experiments of Miss Taistra show that a considerable fall in the CO_2-combining power of the blood takes place as the result of giving hydrochloric acid in this quantity to the dog. There is, however, no great rise of metabolism in consequence.

IX. SODIUM SALT OF GLYCINE.

Lusk (2) has shown that glycine is a powerful stimulant to metabolism. It might be that when administered as a sodium salt its active properties would disappear. Therefore, 9.55 gm. of the substance were dissolved in 300 cc. of water and neutralized by the addition of 10.7 gm. of bicarbonate of sodium. The calculations of metabolism followed the method given by Lusk (7) and the results may thus be summarized:

TABLE VIII.

Dog XIX after Giving 9.55 Gm. of Glycine in 300 Cc. of Water with 10.7 Gm. of $NaHCO_3$ and 2.5 Gm. of Liebig's Extract.

Experiment No.	Date.	Duration of experiment.	R. Q.	Calories.		
				Indirect.	Direct.	Difference.
	1920	*hrs.*				
39	Apr. 22	2	0.84	45.84	45.37*	−0.47

* During the 1st hour, the heat from evaporated water was estimated.

The heat production was 22.94 calories per hour, an increase of 5.31 calories above the basal. This accords with Experiment 61 on Dog III (7) in which administration of 10 gm. of glycine increased the metabolism 4.4 calories.

The specific dynamic effect of glycocoll is therefore in no way diminished by administering it with alkali and the result is far

greater than is achieved after giving the nearly equivalent quantity of 7.6 gm. of glycollic acid, supposedly formed from it by hydrolytic deamination.

These experiments, however, do not exclude the possibility that after all glycollic acid may be the stimulus which increases metabolism, for it has been known since the experiments of Folin and Denis (18) that ingested glycine is absorbed with avidity by the cells of the muscles and liver, and it may be that if deamination takes place within them with the production of glycollic acid, this substance may then play quite a different rôle from that which it plays when brought to the cell from without. Considerations of this sort show to what extent the experimental limitations of the method may play havoc with one's interpretation of the results obtained.

Dakin (19) suggests that glycine reacts upon other amino-acids capable of forming glucose (*e.g.*, alanine), setting them free in the body. These would then become the source of sugar following glycine ingestion and the source of its specific dynamic action. This harmonizes with the fact that 1 molecule of glycine has the same specific dynamic effect as a molecule of alanine, but if 1 molecule of glycine caused the liberation of a molecule of alanine in metabolism in the phlorhizinized dog, then more "extra glucose" would be eliminated than actually takes place.

X. GLUCOSE WITH AND WITHOUT LACTIC AND ACETIC ACIDS.

One of the peculiarities of the specific dynamic action of amino-acids, glucose, and fat is that the extra heat produced by one of the three is specific and that when two or more of the materials are given together the extra heat production is the sum of the influences which each substance given alone would have induced (7). In line with this knowledge it follows that if glucose and lactic acid be given together and lactic acid behaves like a metabolite of alanine, the heat production will be greater than when glucose alone is given, but if it behaves like a metabolite of glucose no appreciable rise will be observed. Also, if acetic acid be given with glucose, one can in like manner analyze whether acetic acid is a normal metabolite of glucose, as it might be if acetaldehyde, supposedly a cleavage product of glucose, were oxidized.

For this purpose the following solutions were given:

A. Glucose, 58 gm. + water, 400 cc. + Liebig's extract, 2.5 gm.
B. Glucose, 50 gm. + lactic acid, 8 gm. + water, 400 cc. + {Liebig's extract, 2.5 gm.
C. Glucose, 50 gm. + acetic acid, 3 gm. + water, 400 cc. + {Liebig's extract, 2.5 gm.

It is evident from Table IX that there is no summation of effect when glucose and lactic acid are given together. Hence ingested lactic acid behaves like a metabolite of glucose and not like one of alanine.

TABLE IX.

Dog XIX after Giving Diets Above Mentioned.

Experiment No.	Date.	State of nutrition.	Duration of experiment.	R. Q.	Calories. Indirect.	Calories. Direct.
	1920		*hrs.*			
47	May 13	Glucose.	2	1.01	44.56	42.17
50	" 20	"	2	1.01	44.71	43.66
Total..			4		89.27	85.83
44	May 10	Glucose + lactic acid.	2	1.07	44.33	42.69
46	" 12	" " " "	2	1.02	44.39	41.29
Total..			4		88.72	83.98
48	May 17	Glucose + acetic acid.	2	0.98	49.59	44.98
49	" 19	" " " "	2	0.97	49.76	45.42
Total..			4		99.35	90.40

Quite different results were obtained after giving acetic acid. Whereas with glucose alone the metabolism was 22.32 calories per hour, after adding acetic acid the heat production rose to 24.84 calories per hour, an increase of 2.52. But it has already been pointed out that after giving 3 gm. of acetic acid alone to this dog the metabolism was increased 3.13 calories.

The results may be thus expressed:

	Calories.	Per cent.
Glucose, 58 gm.	+4.71	+27
Acetic acid, 3 gm.	+3.13	+18
Sum of both.	+7.84	+45
Glucose, 50 gm. + acetic acid, 3 gm.	+7.23	+41

A true summation of effect is revealed similar to that found when glucose and fat are given together, and this supports the hypothesis that acetic acid is a normal intermediate in the metabolism of fatty acid. Since one theory of the production of fat from carbohydrate involves the production of acetaldehyde from methyl glyoxal by CO_2 cleavage and the subsequent polymerization of the aldehyde radicles into fatty acid, and since Lusk (7) has shown that the process of the conversion of fat into carbohydrate does not materially increase the heat production, one may still assume the *possibility* of a production of acetic acid from glucose in metabolism up to the limits of the power of the cell to oxidize acetaldehyde, following which maximum a synthetic production of fat ensues. At this point, however, ingested acetic acid might be oxidized and lift the metabolism to a higher level. It seems, however, more reasonable to believe that, if in the combustion of glucose by the cell acetic acid is produced as a dominant intermediate product, satisfying cell affinities for the oxidation of that substance, then the addition of a small quantity of acetic acid to the diet would not result in a quantitative reaction such as is noted above. Glucose ingestion does not change the CO_2-combining power of the blood (5), therefore no acids are formed in its metabolism which diminish this power.

XI. ALCOHOL CHECKS.

The validity of the data presented rests upon the accuracy of the determinations. The following alcohol checks may reassure the reader. The calculated heat production and the theoretical oxygen consumption were estimated from the carbon dioxide production of the burning alcohol.

TABLE X.

Hourly Average of Alcohol Checks.

Experiment No.	Date.	R. Q.	Error on O_2.	Calories.		
				Indirect.	Direct.	Difference.
	1919		*per cent*			
66	Dec. 12	0.694	−3.9	22.37	22.40	+0.03
67	" 13	0.669	±0	17.47	17.90	+0.43
	1920					
68	Jan. 7	0.674	−1.1	23.32	23.56	+0.23
69	" 13	0.675	−1.1	24.53	24.51	−0.02
70	" 26	0.668	±0	23.70	24.02	+0.32
71	Feb. 3	0.662	±0	18.33	19.26	+1.33
72	" 13	0.632	+5.4	13.59	13.54	−0.05
74	" 17	0.659	+1.2	28.10	28.65	+0.55
77	Mar. 13	0.670	−0.5	27.35	26.60	−0.75
78	" 15	0.666	±0	24.49	24.86	+0.37
80	" 26	0.655	+1.7	22.59	22.55	−0.04
81	Apr. 19	0.684*	−2.5	24.21	23.40	−0.81
82	May 18	0.681	−2.1	26.94	25.66	−1.28
Average per hour..		0.669	−0.2	22.85	22.85	0

* Average of three hourly periods with R. Q. of 0.707, 0.667, 0.675.

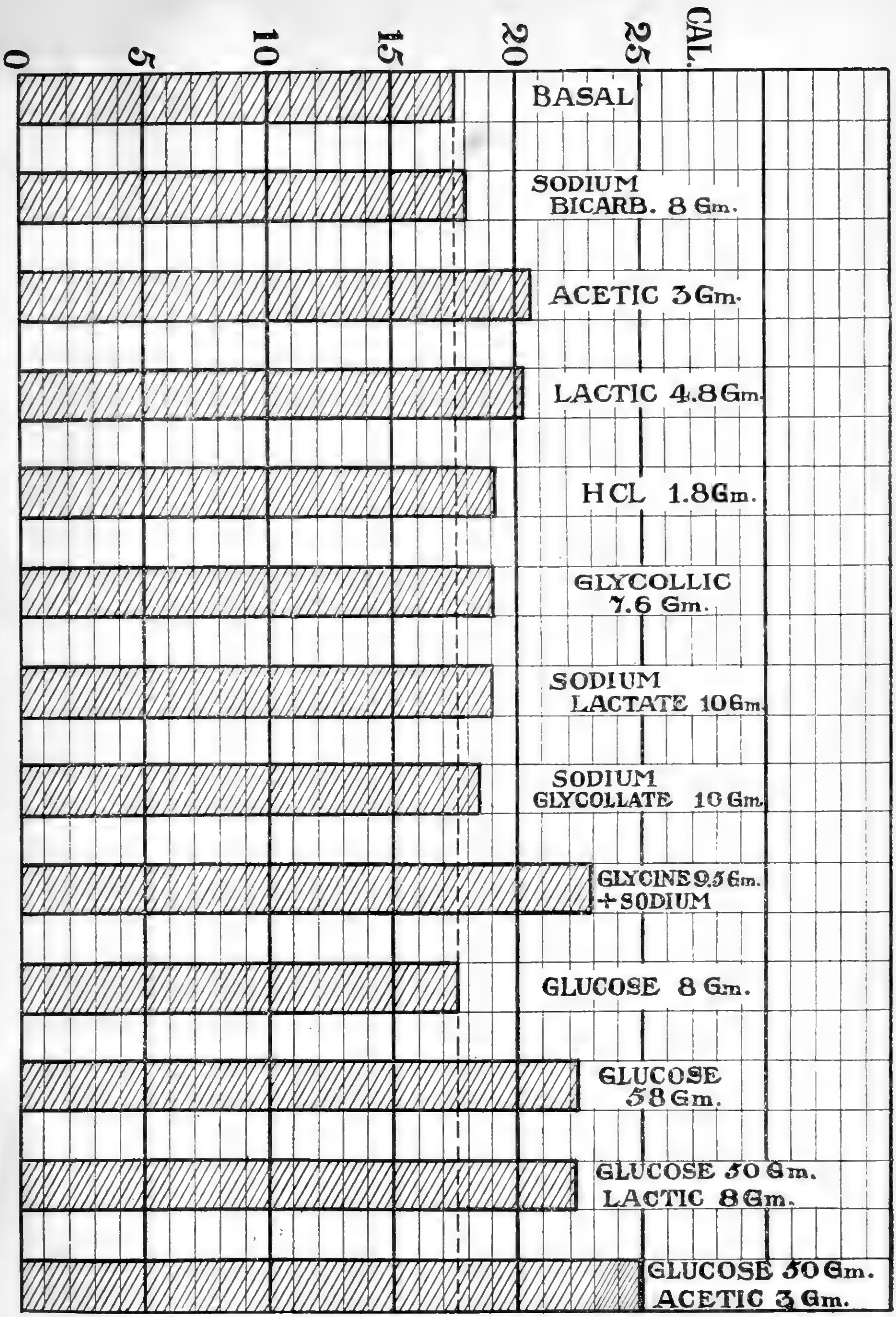
XII. A GENERAL TABULATION.

The results of this work are illustrated in the accompanying chart and are condensed into Table XI.

If one analyzes the experiments in which acids were given one finds no relation between the number of potential hydrogen ions administered and the height of the metabolism, nor is the power to reduce the carbon dioxide-combining power of the blood contributary.

This appears in the following table:

Substance given.	Relative number of potential H ions.	Increase in metabolism.	Decrease in CO_2-combining power of the plasma.
			per cent
Acetic acid, 3 gm.	1.0	3.1	0
Lactic acid, 4.8 gm.	1.2	2.7	14
Glycollic acid, 7.6 gm.	2.0	1.5	28
HCl, 1.8 gm.	1.0	1.6	11


0
5
10
15
20
25
CAL.
BASAL
SODIUM BICARB. 8 Gm.
ACETIC 3 Gm.
LACTIC 4.8 Gm.
HCL 1.8 Gm.
GLYCOLLIC 7.6 Gm.
SODIUM LACTATE 10 Gm.
SODIUM GLYCOLLATE 10 Gm.
GLYCINE 9.5 Gm. + SODIUM
GLUCOSE 8 Gm.
GLUCOSE 58 Gm.
GLUCOSE 50 Gm. LACTIC 8 Gm.
GLUCOSE 50 Gm. ACETIC 3 Gm.

It should be further noted that a quantity of glycine containing only 20 calories induced an increase in metabolism for 2 hours amounting to 5.3 calories per hour, while glucose containing 218 calories, or eleven times as many, raised the metabolism only by 4.7 calories. The glycocoll was neutralized with sodium before ingestion. While one may still speak of amino-acid stimulation in the sense that one speaks of the specific dynamic action of protein, one is still far removed from an actual elucidation of what transpires in the cells to induce the phenomenon.

TABLE XI.

A Summary of All the Experiments.

Nutritive condition.	Calories in diet.	R. Q.	Calories of metabolism per hour.		Number of respirations per minute.	CO_2 in each expiration.
			Indirect.	Above basal.		
						gm.
Basal metabolism	0	0.80	17.6		6	0.016
Liebig's extract	0	0.81	18.1	+0.5	8	0.012
$NaHCO_3$, 8 gm.	0	0.83	18.05	+0.5	7	0.015
Acetic acid, 3 gm	10.5	0.78	20.7	+3.1	8.6	0.013
Lactic acid, 4.8 gm.	17.6	0.84	20.3	+2.7	8.1	0.013
" " 8 " (Na salt)	29.3	0.82	19.0	+1.4	7	0.015
Glycollic acid, 7.6 gm.	16.8	0.94	19.1	+1.5	9	0.013
" " 7.6 " (Na salt)	16.8	0.88	18.5	+0.9	7	0.015
HCl, 1.8 gm.	0	0.84	19.2	+1.6	7.5	0.015
Glycine, 9.5 gm. + $NaHCO_3$, 10.7 gm.	20.1	0.84	22.9	+5.3	10	0.013
Glucose, 58 gm.	217.8	1.01	22.3	+4.7	10	0.014
" 50 " + lactic acid, 8 gm.	217.0	1.04	22.2	+4.6	11	0.014
Glucose, 50 gm. + acetic acid, 3 gm.	198.25	0.97	24.9	+7.2	12	0.013

The administration of these small quantities of materials to a dog is not easy, and the limitation of the method employed appears to me to have been reached in this research. Rubner (20), speaking in the Prussian Academy of Sciences in 1913, said, "Die Wanderung der Stoffe bis zur Zelle zu verfolgen, sie quantitativ zu messen und experimentell zu variieren, das gehört, bis heute wenigstens, zu den unlösbaren Aufgaben. Es ist auch kaum zu

erwarten, dass sich in Bälde die entgegenstehenden Schwierigkeiten überwinden lassen."

It is realized that the methods employed here are relatively crude and the results often merely suggestive.

XIII. SUMMARY.

1. The ingestion of 400 cc. of a broth containing 2.5 gm. of Liebig's extract of beef increased the heat production of a dog from a basal level of 16.1 calories by **0.5** calory per hour.

2. The addition of 8 gm. of sodium bicarbonate to the broth caused no further change in the metabolism.

3. The addition of 3 gm. of acetic acid to the broth caused an increase of **3.1** calories per hour. The material was evidently rapidly consumed.

4. Lactic acid, 4.8 gm. given as before, raised the basal metabolism by **2.7** calories. With 10 gm. of sodium lactate the heat production increased only **1.4** calories per hour, possibly because the alkali favored its transformation into glycogen.

5. Glycollic acid, 7.6 gm., with twice the number of potential H ions contained in it than were present in 3 gm. of acetic acid, increased the metabolism by **1.5** calories or less than half that effected by acetic acid. A like quantity given as glycollate of sodium increased the metabolism **0.88** calory.

6. Hydrochloric acid, 1.8 gm., caused the metabolism to increase **1.6** calories per hour.

7. Glycine, 9.55 gm., containing 20 calories and neutralized with sodium bicarbonate, caused the heat production to rise **5.3** calories per hour. Neutralization therefore did not avail to reduce the activity of the product.

8. Glucose, 58 gm., and glucose, 50 gm., plus lactic acid, 8 gm., manifest exactly the same increases above the basal metabolism, **4.7** and **4.6** calories respectively. Lactic acid therefore behaves like an intermediary metabolite of glucose and not like alanine which would have shown a summation effect.

9. Glucose, 50 gm., plus acetic acid, 3 gm., shows an increase of **7.23** calories per hour. This indicates a summation of the influences of the two factors involved; it is identical with the behavior of glucose and fat when given together; it supports the conception that acetic acid is an intermediary metabolism product

TABLE XII—*General Summa*

Date.	Experiment No.	Time.	CO_2	O_2	R. Q.	H_2O	Calories. Protein.	Non-protein.	Indirect.	Direc
1920			*gm.*	*gm.*		*gm.*				
Jan. 12	4	11.52–12.52	7.02	6.86	0.74	8.05	3.50	18.96	22.46	21.33
		12.52– 1.52	6.52	6.09	0.78	7.21	3.50	16.59	20.09	21.19
									42.55	42.52
" 15	6	11.15–12.15	5.94	4.94	0.87	6.74	3.50	13.16	16.66	17.62
		12.15– 1.15	6.15	5.74	0.78	6.62	3.50	15.43	18.93	19.92
									35.59	37.54
" 16	7	11.11–12.11	6.05	5.46	0.81	6.09	3.50	14.62	18.12	17.82
		12.11– 1.11	5.93	5.60	0.77	6.08	3.50	14.91	18.41	16.56
									36.53	34.38
" 21	10	11.28–12.28	7.38	6.41	0.84	8.08	3.50	17.99	21.49	19.50
		12.28– 1.28	6.24	5.53	0.82	7.21	3.50	14.92	18.42	19.20
									39.91	38.70
" 22	11	11.30–12.30	7.52	6.35	0.86	7.14	3.50	17.92	21.42	19.94
		12.30– 1.30	6.65	5.92	0.82	7.20	3.50	16.22	19.72	20.40
									41.14	40.34
" 23	12	11.44–12.44	6.95	6.07	0.83	8.53	3.50	16.80	20.30	22.15
		12.44– 1.44	6.57	6.40	0.75	8.33	3.50	17.45	20.95	20.62
									41.25	42.77
" 27	13	1.40– 2.40	7.19	6.44	0.81	8.90	3.50	17.95	21.45	19.20
		2.40– 3.40	6.00	5.87	0.74	8.09	3.50	15.68	19.18	19.13
									40.63	38.33
" 28	14	11.23–12.23	6.51	5.87	0.81	8.20	3.50	16.00	19.50	20.63
		12.23– 1.23	6.49	5.85	0.81	7.98	3.50	15.93	19.43	22.78
									38.93	43.41
Feb. 5	19	11.47–12.47	5.91	4.92	0.87	8.49	3.50	13.09	16.59	16.87
		12.47– 1.47	5.52	4.99	0.80	7.76	3.50	13.03	16.53	16.65
									33.12	33.52
" 6	20	11.30–12.30	7.37	6.09	0.88	9.49	3.50	17.13	20.63	14.46
		12.30– 1.30	7.18	5.33	0.98	8.59	3.50	14.88	18.38	17.05
									39.01	31.51

g XIX.

ody temperature.			Morning weight.	Behavior of dog.	Food.
	End.	Difference.			
			kg.		
,	38.06	−0.20	10.60	Quiet. Single movements.	Acetic acid, 3 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.40 a.m.
;	37.76	−0.19	10.57	Quiet. Slight movements.	Basal metabolism.
)	37.90	−0.30	10.57	Moving 1 minute. Quiet.	Meat extract, 2.5 gm.; water, 350 cc. at 10.30 a.m.
l	37.96	+0.05	10.57	Slight movement. Occasional movement.	Lactic acid, 4.8 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.17 a.m.
3	38.21	+0.08	10.55	Slight movement. Single movements.	Lactic acid, 4.8 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.40 a.m.
5	38.14	−0.11	10.52	Quiet. Moving 3 minutes.	Acetic acid, 3 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.21 a.m.
3	38.12	−0.11	10.32	3 slight movements. Quiet.	Acetic acid, 3 gm.; meat extract, 2.5 gm.; water, 400 cc. at 12.35 p.m.
5	38.45	+0.30	10.35	Slight movement. Many slight movements.	Water, 400 cc.; meat extract 2.5 gm. at 10.34 a.m.
:1	38.10	−0.11	10.27	Quiet. "	Basal metabolism.
)7	37.73	−0.34	10.30	Quiet. "	Glycollic acid, 7.6 gm.; meat extract, 2.5 gm,; water, 400 cc. at 10.35 p.m.

TABLE

Date.	Experiment No.	Time.	CO_2	O_2	R. Q.	H_2O	Calories. Protein.	Non-protein.	Indirect.	Dir
1920			*gm.*	*gm.*		*gm.*				
Feb. 9	21	11.20–12.20	6.72	5.93	0.82	8.35	3.50	16.29	19.79	19.9
" 18	22	11.16–12.16	7.22	5.55	0.95	9.93	3.50	15.59	19.09	18.3
		12.16– 1.16	7.09	5.29	0.97	7.86	3.50	14.74	18.24	17.7
									37.33	36.0
" 19	23	12.36– 1.36	5.84	5.12	0.83	7.56	3.50	13.58	17.08	18.36
		1.36– 2.36	5.69	5.19	0.80	7.48	3.50	13.67	17.17	18.74
									34.25	37.1
Mar. 16	24	11.28–12.28	6.26	5.30	0.86	10.23	3.21	14.65	17.86	15.5
		12.28– 1.28	5.42	5.37	0.73	8.97	3.21	14.30	17.51	15.7
									35.37	31.30
" 19	25	11.40–12.40	5.88	5.15	0.83	9.34	3.50	13.68	17.18	17.27
		12.40– 1.40	5.64	5.34	0.77	8.48	3.50	14.04	17.54	16.79
									34.72	34.06
" 20	26	10.57–11.57	6.99	5.46	0.93	9.58	3.50	15.20	18.70	16.42
		11.57–12.57	7.08	5.76	0.89	8.83	3.50	16.07	19.57	17.72
									38.27	34.14
" 27	28	11.00–12.00	6.63	5.63	0.86	10.85	3.50	15.43	18.93	17.52
		12.00– 1.00	6.64	5.80	0.83	9.99	3.50	15.89	19.39	17.39
									38.32	34.9
" 30	29	2.28– 3.28	5.61	5.19	0.78	9.15	3.50	12.10	17.04	17.83
		3.28– 4.28	6.07	6.02	0.73	8.72	3.50	14.62	19.55	18.31
									36.59	36.14
Apr. 6	32	1.06– 2.06	5.82	5.41	0.78	8.22	3.50	14.33	17.83	17.07
		2.06– 3.06	5.65	5.75	0.71	8.27	3.50	15.19	18.69	18.71
									36.52	35 78
" 8	33	12.05– 1.05	6.57	5.75	0.83	9.02	3.50	15.72	19.22	17.94
		1.05– 2.05	6.26	5.70	0.80	8.91	3.50	15.39	18.89	18.35
									38.11	36.2

ontinued.

Body temperature.			Morning weight.	Behavior of dog.	Food.
t.	End.	Difference.			
			kg.		
60	38.48	−0.12	10.27	Moving 3 minutes.	Meat extract, 2.5 gm.; water, 400 cc. at 10.40 a.m.
61	37.44	−0.17	10.05	1 minute slight action. Quiet.	Glycollic acid, 7.6 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.38 a.m.
57	37.67	+0.10	10.05	Slight movement. Restless.	Meat extract, 2.5 gm.; water, 400 cc. at 10.50 a.m.
37	38.05	−0.32	10.02	Quiet. "	Basal metabolism.
97	37.90	−0.07	10.00	Slight movement. " "	Meat extract, 2.5 gm.; water, 400 cc. at 10.50 a.m. Some vomited and water given in place of vomitus.
97	37.78	−0.19	10.01	3 minutes slight action. 2 " " "	Glycollic acid, 7.6 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10 a.m.
19	37.99	−0.20	10.05	Quiet. Slight movement.	HCl, 1.8 gm.; meat extract, 2.5 gm.; water, 300 cc. at 10.11 a.m.
			9.90	Quiet. "	Basal metabolism.
84	37.76	−0.08	9.85	Quiet. "	Basal metabolism.
22	38.05	−0.17	9.80	Quiet. "	Lactic acid, 8 gm.; $NaHCO_3$, 8 gm.; meat extract, 2.5 gm.; water, 400 cc. at 11.17 a.m.

TABLE XI

Date.	Experiment No.	Time.	CO_2	O_2	R. Q.	H_2O	Calories. Protein.	Calories. Non-protein.	Calories. Indirect.	Calories. Direct.
1920			*gm.*	*gm.*		*gm.*				
Apr. 9	34	11.28–12.28	5.99	5.35	0.81	8.91	3.50	14.28	17.78	16.24
		12.28– 1.28	5.49	4.85	0.82	8.13	3.50	12.63	16.13	16.98
									33.91	33.22
" 12	35	12.25– 1.25	5.58	5.15	0.79	8.45	3.50	13.49	16.99	16.87
		1.25– 2.25	5.82	5.38	0.79	8.06	3.50	14.26	17.76	17.93
									34.75	34.80
" 13	36	12.40– 1.40	6.63	5.99	0.81	11.83	3.50	16.39	19.89	18.59
		1.40– 2.40	6.11	5.44	0.82	10.69	3.50	14.60	18.10	17.83
									37.99	36.42
" 20	37	11.38–12.38	7.04	5.73	0.89	10.80	3.50	15.96	19.46	17.13
		12.38– 1.38	6.31	5.43	0.85	9.80	3.50	14.70	18.20	16.26
		1.38– 2.38	6.46	5.25	0.89	9.43	3.50	14.31	17.81	17.52
									55.47	50.91
" 21	38	1.34– 2.34	5.65	5.10	0.81	12.53	3.50	13.40	16.90	16.75
" 22	39	11.16–12.16	8.35	7.18	0.85	6.96(?)*	7.91†	15.82	23.78	(23.94)*
		12.16– 1.16	7.77	6.70	0.84	6.96	7.91†	14.25	22.16	21.43
		1.16– 2.16	6.33	5.85	0.79	6.41	6.51†	12.64	19.15	19.58
									65.09	64.95
May 1	42	10.44–11.44	6.56	5.32	0.90	11.26	3.50	14.56	18.06	19.54
		11.44–12.44	6.22	5.81	0.78	10.49	3.50	15.66	19.16	18.04
									37.22	37.58
" 4	43	1.02– 2.02	5.77	5.09	0.82	14.44	3.50	13.45	16.95	17.72
" 10	44	11.32–12.32	9.14	5.96	1.12	12.71	3.50	17.61	21.11	21.71
		12.32– 1.32	9.36	6.64	1.03	12.33	3.50	19.72	23.22	20.98
		1.32– 2.32	9.12	6.69	0.99	11.81	3.50	19.77	23.27	20.97
									67.60	63.66
" 12	46	11.24–12.24	8.87	6.68	0.97	14.13	3.50	19.64	23.14	21.56
		12.24– 1.24	8.90	6.04	1.07	12.75	3.50	17.75	21.25	19.73
		1.24– 2.24	9.27	6.60	1.02	11.73	3.50	19.57	23.07	22.14
									67.46	63.43

* Water estimated.

† Including amino-acid.

Continued.

Body temperature.			Morning weight.	Behavior of dog.	Food.
rt.	End.	Difference.			
			kg.		
99	37.73	−0.26	9.90	Quiet. “	Meat extract, 2.5 gm.; water, 400 cc. at 10.30 a.m.
35	38.01	−0.34	9.80	Quiet. “	Basal metabolism.
19	38.07	−0.12	9.80	Quiet. “	Lactic acid, 8 gm.; $NaHCO_3$, 7.6 gm.; meat extract, 2.5 gm.; water, 400 cc. at noon.
.55	37.38	−0.17	9.67	Quiet. “ “	Sodium glycocholate, 10 gm.; meat extract, 2.5 gm.; water, 400 cc. at 11 a.m.
.92	37.84	−0.08	9.65	Quiet.	Basal metabolism.
.00*	38.42	+0.42	9.65	Quiet. “ “	Glycine, 9.55 gm.; $NaHCO_3$, 10.7 gm.; meat extract, 2.5 gm.; water, 300 cc. at 10.33 a.m.
.18	38.00	−0.18	9.60	Quiet. 3 minutes moving.	$NaHCO_3$, 8 gm.; meat extract, 2.5 gm.; water, 300 cc. at 10.10 a.m.
.13	37.85	−0.28	9.60	Quiet.	$NaHCO_3$, 8 gm.; meat extract, 2.5 gm.; water, 300 cc. at 11.08 a.m.
.48	38.48	±0	9.55	Quiet. “ “	Glucose, 50 gm.; lactic acid, 8 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.35 a.m.
.04	38.14	+0.10	9.65	3 minutes movement. 3 “ “ Slight “	Glucose, 50 gm.; lactic acid, 8 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.30 a.m.

TABLE XII

Date.	Experiment No.	Time.	CO_2	O_2	R. Q.	H_2O	Calories.			
							Protein.	Non-protein.	Indirect.	Direct.
1920			*gm.*	*gm.*		*gm.*				
May 13	47	11.38–12.38	8.80	6.36	1.01	13.81	3.50	18.66	22.16	21.02
		12.38– 1.38	8.96	6.42	1.02	12.87	3.50	18.90	22.40	21.15
		1.38– 2.38	8.18	6.21	0.96	11.73	3.50	17.95	21.45	21.31
									66.01	63.48
" 17	48	11.05–12.05	9.46	6.93	0.99	12.04	3.50	20.63	24.13	21.76
		12.05– 1.05	9.72	7.35	0.96	12.33	3.50	21.96	25.46	23.22
		1.05– 2.05	7.86	6.42	0.89	11.84	3.50	18.32	21.82	20.15
									71.41	65.13
" 19	49	11.24–12.24	9.57	7.43	0.94	14.18	3.50	22.08	25.58	22.57
		12.24– 1.24	9.52	6.94	1.00	12.99	3.50	20.68	24.18	22.85
		1.24– 2.24	7.56	5.82	0.94	12.01	3.50	16.52	20.02	19.61
									69.78	65.03
" 20	50	11.28–12.28	9.04	6.55	1.00	15.27	3.50	19.32	22.82	22.48
		12.28– 1.28	8.78	6.27	1.02	13.51	3.50	18.39	21.89	21.18
		1.28– 2.28	8.26	5.86	1.03	13.12	3.50	16.96	20.46	19.13
									65.17	62.79

Concluded.

Body temperature.			Morning weight.	Behavior of dog.	Food.
rt.	End.	Difference.			
13			9.70	Quiet.	Glucose, 58 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.40 a.m.
				"	
	38.18			1½ minutes moving.	
		+0.05			
27			9.70	Quiet.	Glucose, 50 gm.; acetic acid, 3 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.05 a.m.
				"	
	38.21			"	
		−0.06			
05			9.70	Restless.	Acetic acid, 3 gm.; glucose, 50 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.25 a.m.
				Quiet.	
	38.33			"	
		+0.28			
97			9.72	3 minutes movement.	Glucose, 58 gm.; meat extract, 2.5 gm.; water, 400 cc. at 10.40 a.m.
				Quiet.	
	38.14			3 minutes movement.	
		+0.17			

of fatty acid by β-oxidation; it renders questionable the idea that acetic acid is an intermediary product of glucose metabolism.

I wish to express my obligation to Miss Sophia A. Taistra who made the urinary analyses and assisted in the entire work.

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ANIMAL CALORIMETRY.

NINETEENTH PAPER.

THE INFLUENCE OF ACIDS UPON THE CARBON DIOXIDE-COMBINING POWER OF THE BLOOD PLASMA.

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The demonstration by Lusk (1) that the ingestion of glycine and alanine caused great increases in the heat production has led to the question, considered in the last paper, whether acid radicles are the cause of those increases in metabolism defined under the head of the specific dynamic action of foodstuffs. In former papers of this series the influence of the concentration of glucose, amino-acids, and of fat in the blood, as bearing upon the intensity of the metabolism of those substances in the cells, has been discussed. This paper deals with the reduction in the quantity of sodium bicarbonate in the blood after administering acid substances to Dog XIX in quantities which are comparable with the amounts which were given to it in the calorimeter experiments described in the last paper. It is known that the reduction in alkali reserve of the blood plasma does not change the actual hydrogen ion concentration of the medium. The method used was to determine the change in the carbon dioxide-combining power of the plasma with the apparatus of Van Slyke.

Van Slyke, Stillman, and Cullen (2) have found in man that, after taking a "mixed diet" at breakfast, the CO_2-combining power of the blood was usually increased, which does not indicate a production of acid metabolites as a cause of the specific dynamic action of the ingested food.

The different substances were always given in a broth containing Liebig's extract of beef. The results may thus be tabulated:

TABLE I.

Influence of Various Metabolites upon the CO_2-Combining Power of the Blood Plasma of Dog XIX.

Date.	Experiment No.	State of nutrition.	Volume of broth given.	CO_2 - combining power of plasma. Start.	Hrs. after the start. 2	4	6	7	Rise or fall at 2nd hr.	Probable rise above basal of 17.6 calories per hr.
1919			*cc.*						*per cent*	*calories*
Oct. 22	1	Liebig's extract, 2.5 gm.	500	56.7	56.0	56.7			−1.2	+0.5
" 24	2	Glucose, 50 gm.	500	58.5	57.0	59.5			−2.5	+4.7
Nov. 1	3	" 50 "	150	59.9	60.1	61.0			+1.7	+4.7
Dec. 22	4	Acetic acid, 2.5 gm.	500	58.5	58.5					+3.1
1920										
Jan. 5	5	" " 3 gm.	400	57.1	58.1	58.1			+1.7	+3.1
Feb. 20	6	Glycollic acid, 7.6 gm.	400	58.4	42.4	41.4	41.0	44.0	−27.6	+1.5
1919										
Oct. 20	7	Lactic acid, 8 gm.	500	58.7	49.0	53.8	53.8		−14.0	+2.7†
1920										
Feb. 20	8	" " 8 "	500	59.5	51.0	56.7			−14.2	
May 22	9	" " 8 "	400	57.6	48.1	56.5	57.5		−16.4	
Mar. 22	10	HCl, 0.8 gm.	400	55.7	47.1	51.9			−15.6	
1921										
May 26	11*	HCl, 1.8 gm.	500	53.5	47.8	49.7	51.6		−10.8	+1.6

* This experiment was performed by Mr. Chanutin.

† Consult also Atkinson and Lusk (3).

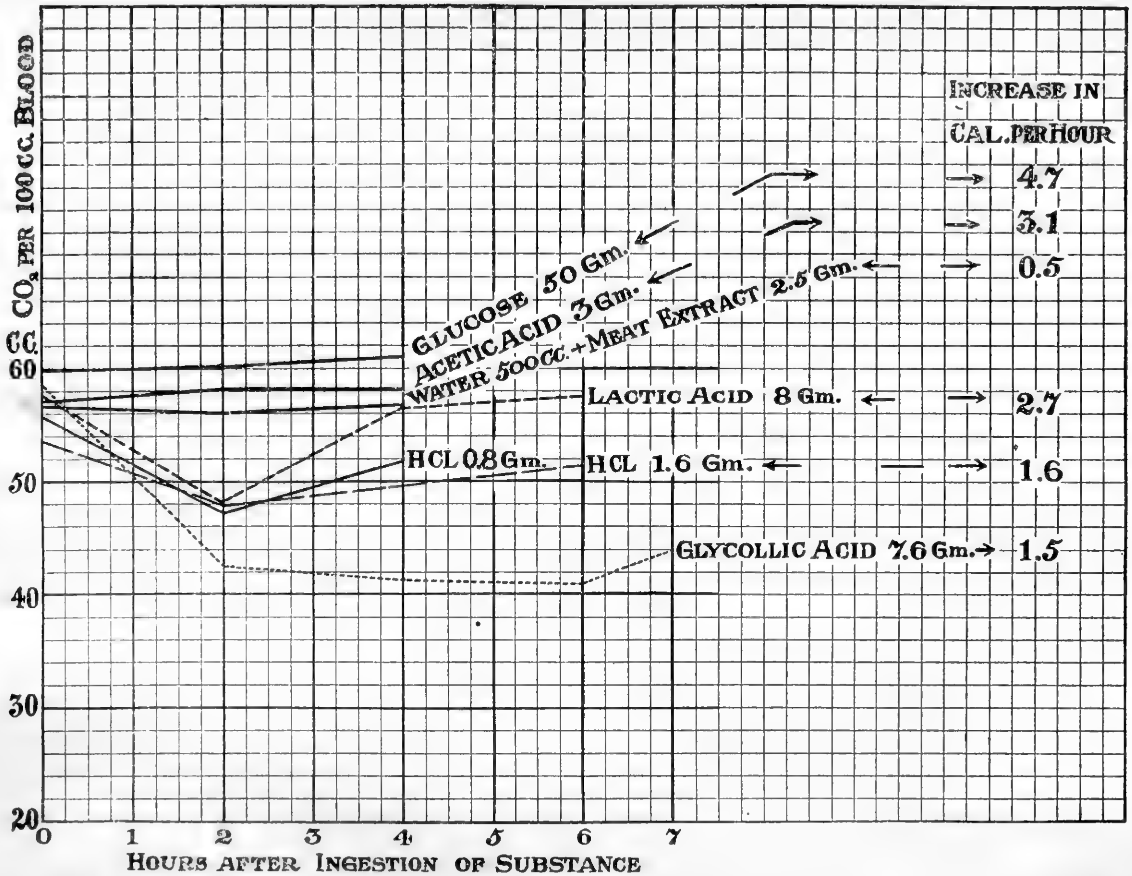

CHART 1. Showing the curves of the carbonic acid-combining power of the blood plasma after giving various metabolites to a dog.

It is evident from this that glucose, 50 gm., and acetic acid, 3 gm., which cause the greatest increases in metabolism, have no effect whatever upon the carbon dioxide-combining power of the blood, whereas glycollic acid, lactic acid, and hydrochloric acid, which have comparatively little influence upon the heat production, produce a profound depression of the CO_2-combining power. The specific dynamic influence of the foodstuffs is therefore not attributable to the introduction of acid metabolites in the blood stream. Chart 1 illustrates these relations.

The absence of any effect upon the CO_2-combining power after giving acetic acid indicates its rapid utilization. The fact that

TABLE II.

Influence of Various Metabolites upon the Acidity of the Urine of Dog XIX.

Experiment No.	State of nutrition.	Volume of broth given.	Urine.		
			Period.	Volume.	0.1 N alkali in cc. per hour to neutralize urine.
		cc.	*hrs.*	*cc.*	*cc.*
1	Liebig's extract, 2.5 gm.	500	5	405	6.3
2	Glucose, 50 gm.	400	4	355	2.6
3	" 50 "	150	4	155	1.8
4	Acetic acid, 2.5 gm.	500	2	390	6.0
5	" " 3 "	400	2	270	6.3
6	Glycollic acid, 7.6 gm.	400	2	245	25.6
7	Lactic acid, 8 gm.	500	6	526	12.3
	" " 8 "	500	4	340	12.3
10	HCl, 0.8 gm.	200	4	145	4.4
	HCl, 1.6 "	300	4	225	11.8

glucose, given in the excess here provided, causes no change in the CO_2-combining power, indicates that no lactic acid can be produced from it in such a manner as to influence by neutralization the alkali reserve of the blood. If lactic acid is formed intracellularly or upon the surface of the cell during the metabolism of glucose, thereby producing a stimulation of metabolism, its effect must be strictly local as it cannot modify the alkaline reserve of the blood. The above experiments demonstrate that lactic acid, if it once enters the blood, may cause a significant fall in the alkali reserve.

In several of the above experiments the urine was titrated to the neutral point, using 0.1 N NaOH against phenolphthalein as indicator.

The results appear in Table II.

The figures here reported merely emphasize the fact that after giving glucose and acetic acid the blood is under no necessity of compensating for a gain in acid radicles by eliminating such through the kidney, whereas in the case of glycollic and lactic acids such a stress is indicated.

CONCLUSION.

The specific dynamic action of the foodstuffs is not dependent upon the level of the alkaline reserve of the blood plasma as measured by its CO_2-combining power.

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ANIMAL CALORIMETRY.

Twentieth Paper.

THE INFLUENCE OF THE INGESTION OF MEAT AND OF GLYCINE AND ALANINE UPON THE CARBON DIOXIDE-COMBINING POWER OF BLOOD PLASMA.

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(Received for publication, October 13, 1921.)

The problem is but an extension of that presented in the last two papers and may be stated in the words: Do the amino-acids formed after the ingestion of meat by a dog so reduce the alkali reserve of the blood that this becomes a contributing cause of the great increase in heat production known as the specific dynamic action of meat? Hasselbalch (1) observed a lower CO_2 tension of the alveolar air after administering a meat diet to a man than

TABLE I.

Influence of Meat, Glycine, and Alanine Ingestion upon the CO_2-Combining Power of the Blood Plasma of the Dog.

Date.	Experiment No.	State of nutrition.	CO_2-combining power of 100 cc. of plasma.						
			Start.	Hrs. after the start.					
				1	2	3	4	5	6
1920				*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Sept. 27	1	Beef, 940 gm.	59.8		68.7		63.0		63.4
Oct. 5	2	" 810 "	64.8	71.3	61.5		63.0	64.6	65.6
" 14	3	" 1,000 "	61.9	63.8	64.8	69.4	68.4	67.5	61.0
1921									
May 21	4	" 1,000 "	65.0	65.5	69.4		70.3		69.4
" 11	5	Glycine, 15 gm.	58.4	63.3	64.2		62.2		
" 13	6	" 15 "	62.9	69.6	64.7		60.9		
Apr. 13	7	Alanine, 15 gm.	65.7	69.5	67.6				
May 9	8	" 15 "	65.9	67.8	67.8		66.8	65.0	

after giving an alkaline vegetarian diet, which might be interpreted as signifying that the latter caused a greater increase in the alkaline reserve than was brought about by the meat diet.

The amino-acids were administered as heretofore in a warm broth containing 2.5 gm. of Liebig's extract and 500 cc. of water.

It is apparent from Table I that there is always an increase in the CO_2-combining power of the blood following the ingestion of meat, of glycine, or of alanine. The broth itself was without influence.

It is possible that the interpretation of Erdt (2) is correct, that the rise in the CO_2 tension in the alveoli which follows the taking of a meal is due to the secretion of hydrochloric acid by the gastric juice.

CONCLUSION.

It is apparent that the ingestion of meat or of amino-acid derivatives of meat may materially increase the alkali reserve of the organism of a dog, as measured by the CO_2-combining power of the blood plasma. Since the administration of bicarbonate of sodium has no influence upon the heat production of a dog (Paper 18), it is evident that the great increase in the heat production which takes place during the hours immediately after the ingestion of meat in not determined by any change in the alkaline reserve of the blood, nor can such change be interpreted as being even a participating element in the causation of the phenomenon.

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THE MICRO DETERMINATION OF CALCIUM IN WHOLE BLOOD, PLASMA, AND SERUM BY DIRECT PRECIPITATION.

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Methods for the determination of calcium, if classified according to the manner of obtaining solutions from which the calcium may be precipitated, fall into three groups: (1) Destruction of the organic matter (proteins) by ashing or by digestion with an oxidizing mixture and solution of the ash in hydrochloric acid (1–8). (2) Precipitation of the proteins, as by picric, tungstic, or trichloroacetic acids, and use of aliquot part of the filtrate (9, 10). (3) Direct precipitation, in the presence of proteins, so far only applied to whole blood, plasma, and serum (11–15).

Although every method utilizes the oxalate ion for the precipitation of calcium, considerable variation is observed in the manner of obtaining the H ion concentration at which pure calcium oxalate is most completely precipitated. After obtaining the washed calcium oxalate the following procedures have been used for the actual estimation of the calcium:

1. Gravimetrically, by conversion to CaO or $CaSO_4$ and weighing (1, 2, 11).

2. Volumetrically; (*a*) by titration with potassium permanganate (3, 6–8, 10, 12–15) and, (*b*) by solution in excess acid and titration of excess acid (5).

3. Nephelometrically, by conversion into a calcium soap (9).

4. Colorimetrically, by decolorization of ferric thiocyanate with oxalates (4).

5. Iodometrically, by solution of CaO in excess HCl, the excess acid then determined by estimating the amount of iodine liberated (5). $5\ I^- + IO_3^- + 6\ H^+ \rightarrow 3\ H_2O + I_2$.

In 1917 the writer began some investigations on calcium metabolism[1] in which it was desirable to determine calcium in small amounts of whole blood and plasma. Although several micro methods for the determination of calcium in blood and other biological fluids were then available in the literature it soon became evident from experiments that the results obtained by the different methods showed large variations. Because of this lack of agreement in results the writer decided to make a general survey of several of the most promising methods. In order to make a fair comparison it was necessary to acquire certain technique for each method; this made the task very tedious and involved a large number (more than 2,000) of calcium determinations.

In the analysis of biological material the destruction of organic matter by ashing the sample in platinum, although a very old procedure, is still much used, and is commonly taken as the basis in establishing the accuracy of new methods. In ashing blood samples the loss of calcium occurs principally through: (1) the expulsion of small particles by the rapidly evolved gas, during the early stages of incineration; and (2) failure to dissolve completely the fused residue remaining after the destruction of the organic matter. It is necessary to digest the residue in hot hydrochloric acid (6 N) for approximately 1 hour to insure complete solution. Aside from the difficulties of ashing and subsequent solution, the remaining objection to this method is the amount of time required (evaporation, ignition, solution, filtration). However, if carried out in a way to minimize the losses, the method undoubtedly affords accurate results, and in the experimental work of this paper has been taken as the standard with which the results of other methods were compared.

While the destruction of organic matter with hot, concentrated nitric acid according to the method of Marriott and Howland (4) gave fair results (see Table I), the method has no advantages over ashing in platinum and has the following additional disadvantages: (1) It is more difficult to prevent mechanical losses during digestion, as most samples, especially whole blood, foam abundantly. (2) The digestion in glassware, even of the best grade,[2] is a questionable procedure, where such small amounts of calcium are

[1] Unpublished work.

[2] The small Pyrex flasks were all etched during the digestion.

to be determined. (3) The increased time required to complete the digestion and evaporate the excess acid, 3 or more hours being necessary for this one step.

In beginning the experimental work on calcium, Lyman's (9) nephelometric method was selected, because of the apparent rapidity with which determinations could be made. A large number of determinations made[3] on solutions of known calcium content (*i.e.*, different strength standard solutions of calcium nitrate prepared according to Lyman's directions) gave such discordant results that but few determinations were made on blood samples.

TABLE I.

Determination of Calcium after Destruction of Proteins by Digestion with Nitric Acid—Also the Recovery of Added Calcium.

Ca present in sample.	Amount of Ca added.	Total Ca.		Added Ca recovered.	
		Present.	Determined.*		
Horse plasma, 5 cc. samples.					
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
0.576					
0.567					
0.584					
0.577					
0.564					
0.591					
0.555					
	0.396	0.969	0.955	0.382	96.5
	0.264	0.837	0.834	0.261	98.8
	0.352	0.925	0.940	0.367	104.2
0.587	Sample ashed in platinum for comparison.				
Average....0.573					

* The calcium was precipitated according to the Halverson-Bergeim (10) modification of McCrudden's (24) method.

† Calculation based on the assumption that the amount of calcium added was 100 per cent accurate.

[3] These determinations were made in 1917 by H. A. Mattill and the writer, Department of Nutrition, University of California. The work was arranged so that one of us made up the unknowns and standards, and the other made the nephelometric readings.

Lyman (9) emphasizes the clarity of the ammonium stearate reagent[4] as a prerequisite to the successful use of the method, but neglects to take consideration of other possible sources of error:

(1) The precipitation of the blood proteins is made in too small a volume (a total of 20 cc.) which, through slight variations in the rate of adding the blood to the trichloroacetic acid, influences the distribution (by varying the amount occluded and adsorbed) of calcium. It was thought possible to prove this point through a series of analyses made on the regular aliquot, the remaining filtrate, and the precipitate, but most of the work along this line was rendered valueless because it was necessary to conduct the ashing of the protein precipitates in either porcelain or silica dishes.[5] The data presented in Table II include only those determinations in which the protein precipitates were ashed in platinum. It is also evident from data presented in Table IX that the highest calcium values are obtained from filtrates prepared by this method, and also that dilution (to 50 cc.) gives results which compare favorably with those obtained by the standard method (*i.e.*, ashing in platinum).

(2) Since the number of colloidal particles present in a given volume forms the basis for the actual determination by this method and since the stability of colloidal suspensions in general is altered by changes in the H ion concentration it would appear that more emphasis should be placed on the maintenance of a more definite H ion concentration. The calcium stearate solutions are poorly buffered, and relatively small variations in the addition of the nitric acid (in amount or in normality) would appreciably change the true H ion concentration and thereby modify the colloidal calcium stearate particles, both as to numbers and size. It was pointed out by Halverson, Mohler, and Bergeim (16), and more recently by Kramer and Howland (8) that the normal values obtained by this method show unusually large variations.

[4] Three different lots of this reagent were prepared (all with tested Kahlbaum chemicals), and the last one, made up in November, 1917, is clear at the present writing.

[5] A platinum dish was lost in ashing the precipitates of deproteinized whole blood (iron and phosphorus?). Ashing in porcelain or silica is undesirable since the alkaline phosphates attack these dishes and make it very difficult to dissolve the fused residue. With porcelain considerable extra calcium is recovered.

TABLE II.

*Determination of Calcium in Trichloroacetic Acid. Filtrates and Precipitates.**

Volume of aliquot taken.	Volume of plasma represented by the aliquot.	Ca determined in aliquot.†	Ca determined in remainder of sample. Filtrate and washings.†	Ca determined in remainder of sample. Precipitate.†	Total Ca recovered from complete sample.	Ca per 100 cc. of plasma. Calculated from aliquot.	Ca per 100 cc. of plasma. Calculated from determination of complete sample.
Horse 1, Plasma sample I.							
cc.	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
10	2.5	0.269	0.231	0.054	0.554	10.76	11.08
10	2.5	0.261	0.251	0.054	0.566	10.44	11.32
16+‡		0.444	0.120§	0.020	0.584		11.68
16+‡		0.437	0.120§	0.018	0.575		11.50
					0.570§		11.40
					0.568§		11.36
Horse 3, Plasma sample I.							
10	2.5	0.390	0.222	0.065	0.587	12.00	11.74
					0.606‖		12.12
					0.606‖		12.12
Rabbit "M," Plasma sample I.							
10	2.5	0.322	0.205	0.089	0.616	12.88	12.32
10	2.5	0.332	0.237	0.108	0.677	13.28	13.54
					0.613		12.26
Rabbit "M," Plasma sample II.							
10	2.5	0.283	0.201	0.072	0.556	11.32	11.12

* Deproteinized exactly as described by Lyman (9).

† The calcium was precipitated according to the Halverson-Bergeim (10) method.

‡ Represents all of the filtrate.

§ Washings only.

‖ Samples ashed in platinum, residues dissolved in hot (6 N) hydrochloric acid, evaporated to small volume (1 to 2 cc.), and the calcium precipitated by the Halverson-Bergeim method (10).

Subsequent to the abandonment of the nephelometric method the writer developed a procedure[1] which was, except for the use of picric acid as protein precipitant, practically identical with that published by Halverson and Bergeim (10). Although these authors state that picric acid may be sufficiently purified for their procedure by recrystallization from hot water, solutions prepared from different lots of picric acid purified in this manner exhibit marked variations in the shade and intensity of color, and as the deeper color makes it more difficult to observe the changes of indicators during neutralization, it is desirable to purify this reagent according to the method of Folin and Doisy (17). In using the Halverson-Bergeim procedure (10) the writer found that the use of alizarin (alizarin monosodium sulfonate) gave high results when the calcium was determined by titration with potassium permanganate. Relatively large amounts of this dye are carried down[6] when centrifuging and, although a water-soluble substance, the amount adsorbed by the washed calcium oxalate is sufficient to materially increase the amount of permanganate. A few trials showed that acid solutions of alizarin are readily oxidized by potassium permanganate (0.05 cc. of a 1 per cent alizarin solution + 5 cc. of N sulfuric acid heated to 75°C. required 0.65 cc. of 0.01 N potassium permanganate. 5 cc. of the same acid and at the same temperature require 0.05 cc. of the permanganate). Methyl red (*o*-carboxybenzeneazodimethylaniline) is very satisfactory as an indicator and is not carried down with the calcium oxalate. The tungstic acid method (18) of deproteinization should not be used if calcium is to be determined in the filtrate. Experiments show that the calcium values obtained from these tungstic acid filtrates are much lower (by approximately 35 per cent) (see Table III). Heating the precipitated mixture in a water bath for 2 or 3 minutes gives still lower calcium values (see Table IX). It has been shown by the experiments of Rona and Takahashi (19), Cushny (20), von Meysenbug, Pappenheimer, Zucker, and Murray (21) that from 25 to 40 per cent of the total calcium (horse, beef, pig, dog, man) is present as a non-diffusible compound. It is possible that the low calcium values obtained after deproteinization with tungstic acid may be due to the low H

[6] Several lots of this indicator were prepared (0.2 per cent solution) and all were equally adsorbed.

ion concentration of the precipitated mixture (pH 3.0), a condition that may either be unfavorable to the dissociation of the calcium-protein compounds already present or favorable to formation of additional calcium-protein compounds. The influence of H ions is apparent when other methods of deproteinization are considered, since the picric acid and trichloroacetic acid mixtures possess a concentraton of H ions sufficient to form soluble calcium salts. Although the total amounts of tungstate (380 mg. of WO_4) and sulfate (160 mg. of SO_4) in the mixture are large in comparison to the amount of calcium (0.5 mg. of Ca) it is highly improbable that these are in any way responsible for the calcium values (the solubility of either $CaSO_4$ or $CaWO_4$ in distilled water is approximately 2 gm. per 1,000 cc.[7]).

Method.

Whole Blood.—With a pipette place 5 cc. of citrated whole blood[8] in a 25 cc. volumetric flask, with the same pipette add two 5 cc. portions of warm water (about 65°C.), mix, and allow to stand 20 minutes or longer. Add 5 cc. of 1 per cent ammonium chloride,[9] make up to volume with distilled water, mix thoroughly, and transfer to a 50 cc. centrifuge tube. Cover the tube with rubber dam or paraffined paper (to prevent evaporation and to keep out dust) and centrifuge at high speed for 20 minutes.[10] With a pipette remove 15 cc. or if possible 20 cc., transfer to a 50 cc. centrifuge tube,[11] and while rotating the tube to agitate the

[7] Figures taken from Van Nostrand's Chemical annual, New York, 3rd edition, 1915.

[8] 1 cc. of saturated sodium citrate solution, pH 7.4, (approximately 900 mg.) per 100 cc. of whole blood. This is the minimum for most blood samples.

[9] The necessity of this reagent is not completely proved. The presence of the additional electrolyte seems to facilitate the sedimentation of the light stroma when the mixture is centrifuged.

[10] The deep red supernatant liquid must be clear. To prevent dispersion of the light stroma the centrifuge must slow down very gradually.

[11] The tubes obtained in the market are not satisfactory, as they do not stand repeated centrifuging. (The tubes are drawn out too far, making very thin walls, and many are not properly annealed.) The writer is indebted to Mr. W. J. Cummings, of the College of Chemistry, for making special Pyrex tubes, see Fig. 1.

liquid slowly add 4 cc. of 3 per cent ammonium oxalate, mix thoroughly, and allow to stand over night. Rub down walls of the tube with a rubber policeman (washing the policeman with a small amount of distilled water), and centrifuge at moderate speed (1,800 R.P.M.) until clear, usually 5 minutes is ample time. Completely remove the supernatant liquid by means of a siphon, stir up the precipitate with a fine stream of cold, distilled water, wash down the walls of the tube, using in all approximately 35 cc. of water. Centrifuge immediately and completely siphon off the wash water. Dissolve the precipitate in 5 cc. of approximately normal sulfuric acid, heat to 75°C., and titrate with 0.01 normal potassium permanganate.[12]

Plasma (or Serum).—Place 1 to 5 cc. of citrated plasma (or serum)[8] in a 50 cc. centrifuge tube and, while rotating the tube, slowly add 3 per. cent ammonium oxalate, equal in volume to one-half the amount of serum or plasma. Mix thoroughly and allow to stand over night. The remainder of the procedure is the same as that described for whole blood.

Reagents.

Potassium Permanganate, Approximately 0.01 N.—After dissolving the salt in the proper amount of water the solution should be heated on a steam bath for 36 to 48 hours, or allowed to stand at room temperature 10 days or more (22). The solution is then filtered through asbestos[13] and stored in amber bottles, well pro-

[12] In the titration of calcium oxalate obtained by direct precipitation the end-point is not as permanent as in the case of pure inorganic solutions and the amount of permanganate necessary for the blank is greater (especially true with whole blood). The presence of traces of organic matter accounts for the above conditions and in order to decide upon a reproducible end-point it is advisable for each operator to make a series of preliminary determinations. The blanks are determined from samples of whole blood and plasma which have been treated according to the regular procedures except that no oxalate is added. The writer found the following averages for blank determinations: (1) Whole blood = 0.10 cc. of 0.01 N potassium permanganate; (2) plasma = 0.08 cc. of 0.01 N potassium permanganate; and (3) sulfuric acid (5 cc. of N) = 0.05 cc. of 0.01 N potassium permanganate.

[13] In order to avoid the reducing action of organic matter the asbestos should be digested with aqua regia and washed free from chlorides.

tected from dust. The standardization is best made with sodium[14] or calcium oxalate. The oxalates are dissolved in and made up to volume with approximately N sulfuric acid. Small portions (5 to 10 cc.) are then measured into 50 cc. centrifuge tubes, heated to 75°C., and titrated. These acid oxalate solutions, if carefully protected from dust, etc., may be kept for several months (23) and used for subsequent titrations.

Ammonium Chloride, a 1 Per Cent Solution.—This reagent should be tested for calcium as follows: 10 gm. are placed in a platinum dish and sufficient heat is applied to volatilize the ammonium chloride. The ash is dissolved in a minimum amount of hot 6 N hydrochloric acid (previously tested for calcium) and the calcium precipitated by a modification[15] of McCrudden's (24) method.

Sulfuric Acid, Approximately N.—Add 28 cc.[16] of concentrated C.P. sulfuric acid to 970 cc. of distilled water.

In any micro method it is necessary to test carefully the reagents used and this is especially true when small amounts of such common substances as calcium, sodium, etc., are to be determined. Since many of the calcium methods make use of some modification of McCrudden's (24) procedure, the following directions are given for the testing and preparation of the necessary calcium-free reagents:

Sodium Acetate, a 20 Per Cent Solution.—If several lots of this reagent are available make qualitative tests and select the sample showing the smallest amount of calcium.[17] Dissolve the salt (200 gm. of the crystalline salt in a final volume of 1,000 cc.) in nearly the required amount of distilled water, add 0.5 per cent of ammonium oxalate (this salt should be dissolved in a minimum amount of water), and bring to volume. If convenient place in a refrigerator (10–12°C.) and allow to stand 48 hours, stirring thoroughly once or twice each day. Centrifuge, or filter through

[14] Consult *Circular 40, U. S. Bureau of Standards, Dept. Commerce,* 3rd edition, 1920. Standard samples of sodium oxalate may be obtained from the Bureau of Standards.

[15] The writer has made use of the Halverson-Bergeim modification (10).

[16] The 50 cc. of concentrated acid used by Kramer and Tisdall (14) is too much.

[17] The writer found calcium in several different lots of this salt.

a doubly washed (HCl and HF) paper, and store in parraffin-coated bottles.

Dilute Hydrochloric Acid.—Test the concentrated acid by evaporating 25 cc. in a platinum dish. Evaporate almost to dryness (leave 1 small drop), dilute to a small volume (5 cc.), and precipitate the calcium in the regular way.[15]

Ammonium Hydroxide, Approximately 2 N.—The writer found calcium in many samples of C.P. ammonia.[18] It is best to prepare this reagent by passing pure ammonia gas into conductivity water. During this operation the water should be kept at a low temperature (pack container in ice) and sufficient space allowed in the receptacle for the increase in volume. Determine the strength by titration, and dilute to the desired normality. Store in paraffin-coated containers.

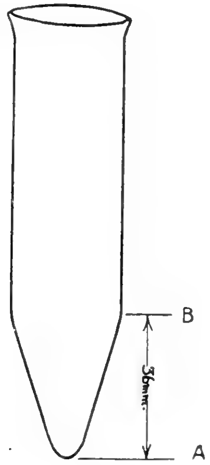

FIG. 1.

Apparatus.

Since the centrifuge has become such a generally useful instrument practically every laboratory is equipped with one or more.

[18] In testing concentrated ammonia for calcium it is best to evaporate 25 cc. in platinum. Tests for calcium after dilution are often negative since a much smaller amount of calcium is present and that which is present does not completely precipitate because of the excess of ammonium salts.

Aside from this instrument very little is required in the way of special apparatus.

Burette, Capacity 10 Cc.—Graduated in 0.02 cc., and allowing for estimation of 0.01 cc. to meet the requirements of the Bureau of Standards.[19]

Centrifuge Tubes, Capacity 50 Cc.—Best annealed Pyrex glass. See Fig. 1.

Siphon.—A very satisfactory one is described by Halverson and Bergeim (10).

DISCUSSION.

It has been generally supposed that it is impossible to obtain the total calcium of serum or plasma by direct precipitation with oxalate, the definite statements to this effect being apparently based on the diffusion experiments of Rona and Takahashi (19), in which it was found that from 25 to 35 per cent of the total calcium of different sera (horse, pig, beef) was present in a non-diffusible state. The more recent investigations of Cushny (20) and of von Meysenbug, Pappenheimer, Zucker, and Murray (21) show approximately the same amount (25 to 40 per cent) of non-diffusible calcium in beef, dog, and human sera. If it be granted from the results of these different diffusion experiments and from the experiments of Pauli and Samec (25), that it is possible for calcium-protein compounds to exist, and if we accept the assumption that much of the blood calcium is present in the form of the bicarbonate (Loew, 26; Rona and Takahashi, 19; Brinkman, 27), then the following equilibria would be involved:

$$\frac{(Ca^{++})\ 2(HCO_3^-)}{Ca(HCO_3)_2},\quad \frac{(H^+)\ (HCO_3^-)}{H_2CO_3},\quad \frac{(Ca^{++})\quad (\text{protein ion})}{\text{calcium-protein}}.$$

If Ca ions were removed from serum, as by dialysis or precipitation, one should then expect a progressive shifting of equilibria until finally all of the calcium bicarbonate and the calcium-protein had dissociated. The few experiments on record (21) indicate that small increases in the H ion concentration, from pH 7.6 to 7.0 (by changing the CO_2 tension), have no effect upon the amount of diffusible calcium. However, the same experiments show that

[19] Such burettes are obtainable from Emil Greiner and Co., New York.

if serum be dialyzed for several days against an isotonic, calcium-free solution there is a slow diffusion of calcium which would indicate dissociation of the calcium-protein complex.

Přibram (11), in 1871, was the first to report the direct precipitation of the total calcium from serum. After adding an excess of ammonium hydroxide to the sample (100 cc.) ammonium oxalate was added and the mixture allowed to stand for about 30 minutes. The calcium oxalate was obtained by centrifugalization and the calcium finally determined by weighing as CaO. Gerlach (28) examined some precipitates obtained by this procedure and found them contaminated with phosphorus and magnesium, results which undoubtedly caused the method to be regarded with suspicion and led to the common belief that calcium could not be completely precipitated by direct addition of oxalate to serum. Lyman (9) was unable, by addition of powdered potassium oxalate to the whole blood, to precipitate completely the calcium from beef and cat sera. In 1919, de Waard (12) described a method for the direct precipitation of calcium from serum. Both Přibram (11) and de Waard (12) compare the calcium values obtained by direct precipitation with those obtained by ashing the serum on platinum. If the values obtained by ashing represent 100 per cent of the calcium then the errors of the respective determinations are as follows:

Přibram; +1.7, +6.0, +13.3, −1.2, −2.6, +6.5, +9.6 per cent.
De Waard; +1.1, −0.4, +0.6 per cent.

Since making the preliminary report (13) on the direct precipitation of calcium, the writer has found by experiment that the dilution of plasma (or serum) with water or 1 per cent ammonium chloride was unnecessary,[20] and in the procedure outlined in this paper, the 3 per cent ammonium oxalate solution is added directly to the plasma (or serum). After dilution of the serum with approximately an equal volume of water, Kramer and Tisdall (14) added sulfuric acid, ammonium chloride and, following the addi-

[20] The precipitated calcium oxalate has been repeatedly examined for the presence of phosphorus (after oxidation with hot HNO_3), and in no case was the test definitely positive. It is somewhat more difficult to prove the absence of magnesium. It has been done indirectly in the experiments (see Table III) where magnesium was added to the plasma.

tion of oxalic acid, a small amount of sodium acetate. This procedure would indicate that these authors had attempted to follow the details of the macro method as outlined by Fresenius (29), Richards, McCaffrey, and Bisbee (30), and McCrudden (24), all of whom state that the precipitation of calcium should be made in solutions containing ammonium chloride and in the presence of relatively high concentration of H ions, traces of calcium being finally precipitated by addition of OH ions. Kramer and Tisdall have, in a very recent publication (15), simplified their procedure so that after dilution of the serum with water the calcium is precipitated by addition of saturated ammonium oxalate.

Where calcium is to be determined by titration with permanganate it is necessary to remove all adsorbed oxalates, but in the micro method the number of washings is also limited, because of the appreciable solubility of calcium oxalate in water. According to Richards, McCaffrey, and Bisbee (30), who made a number of determinations at different temperatures, the solubility of calcium oxalate at 25°C. is 7 mg. per 1,000 cc. Kohlrausch and Rose (31) report 5.9 mg. per 1,000 cc. at 18°C. This would mean that if one were working with 0.4 mg. of Ca, which is as much as would be found in 3 cc. of any normal plasma, and used 50 cc. of wash water (assuming the wash water to be completely saturated with respect to calcium oxalate), the loss of calcium would be approximately 22 per cent. [6.5 mg. (the average) $\times$ 0.27 (the factor) $=$ 1.76 mg. of Ca per 1,000 cc. 0.00176 (mg. per cc.) $\times 50 \div 0.4 \times 100 = 22$.]

The separation of precipitates from the mother liquors and washing fluids by centrifugalization is very thorough and if the supernatant fluids are completely removed much less washing is required. To facilitate this washing and subsequent removal of wash water the writer has made use of a specially shaped centrifuge tube, see Fig. 1. The round bottom centrifuge tube suggested by Halverson and Bergeim (10) is undesirable because the precipitate is spread over a much larger surface and it is not possible to completely remove the supernatant fluid without disturbing the precipitate. The tube used by de Waard (7) is also undesirable because one must use a capillary pipette for complete removal of the supernatant liquid, and, still more important, it does not readily permit suspension of the precipitate in the wash water.

After trying various ways,[21] only one washing of the calcium oxalate is included in the author's method. To minimize the solution of calcium oxalate the wash water should be cold, not over 10°C., and must be removed (by centrifugalization) as quickly as possible, not over 15 minutes. To insure complete removal of excess oxalate the precipitate must be thoroughly stirred up and the sides of the tube well washed. If a number of samples are to be washed it is convenient to have a pressure wash bottle (either gravity or compressed air) with a fine outlet. If, for one reason or another, a second washing is necessary, it should be carried out as quickly as possible. The writer has often titrated portions of the wash water (first washing) and finds the following results are typical:

5 cc. of wash water + 5 cc. of sulfuric acid require 0.13 cc. of $KMnO_4$.
5 " " " " + 5 " " " " " 0.14 " " "
10 " " sulfuric acid require 0.06 cc. of $KMnO_4$.

Assuming that 0.1 cc. (2 large drops) of wash water remain after siphoning, the amount of 0.01 N permanganate required would be approximately 0.002 cc.

In the various quantitative procedures (22, 23, 28) for calcium it is customary to allow the solutions to stand several hours to insure complete precipitation of *traces* of calcium. It is reasonable to suppose that these so called traces exist in the micro determinations and that they constitute a greater percentage of the total calcium where, as in the case of 3 to 5 cc. of blood, less than 0.5 mg. of calcium is present. In order to determine the conditions necessary for complete precipitation by the direct method the writer made a number of calcium determinations to show the effects of the following variations upon the time required and upon the amount of calcium recovered: (1) Precipitation from unaltered plasma; (2) dilution of plasma with water; (3) dilution of plasma with 1 per cent ammonium chloride; (4) addition of excessive amounts of various salts ($NaCl$, NH_4Cl, $(NH_4)_2SO_4$, Na_2SO_4, etc.); and (5) a large excess of magnesium (ratio of Ca : Mg = 1 : 11), both in the presence and absence of ammonium chloride.

[21] At different times the calcium oxalate was washed with: (1) 0.5 per cent ammonium oxalate followed by a second washing with distilled water; (2) 0.1 N ammonium hydroxide; and (3) 0.9 per cent sodium chloride. See Halverson and Bergeim (10), p. 166.

From the results of these experiments, presented in Tables III and IV, it is evident that: (1) The time required for complete precipitation from unaltered plasma is over 3 hours, which is contrary to the reports of Přibram (11) and de Waard (12). In this connection it may be mentioned that many of the micro methods (1, 5, 6, 9, 10) require 4 hours or more for complete precipitation of calcium from protein-free solutions. (2) While the dilution of plasma with water (2 to 3 volumes) apparently allows a more rapid precipitation of the calcium, the reaction is not complete in the short time (30 minutes) allowed by Kramer and Tisdall (14, 15). This point was not completely proved because the dilution decreased the concentration of citrate (the minimum amounts being used) to such an extent that a small amount of fibrin formed in many of the samples. (3) Dilution of the plasma with equal amounts of 1 per cent ammonium chloride or the presence of ammonium chloride or sulfate in appreciable amounts (900 to 2,300 times the amount of Ca) greatly retards the rate of precipitation, the effects being most pronounced with the sulfate. It is also evident that when these solutions stand over night, the presence of ammonium chloride has no effect upon the final calcium values, while with the sulfate there is a definite loss of calcium. (4) The addition of large amounts of magnesium (1 Ca : 11 Mg) to plasma has no effect upon the calcium values and contrary to the usual inorganic procedures for the separation of calcium in the presence of magnesium (22, 23, 28, 29) ammonium chloride is not necessary in the direct precipitation from plasma.[18]

Although potassium permanganate has often been utilized in the quantitative estimation of calcium (3, 6–8, 10, 12–15), Krüger (32) is the only one to record any data to indicate the accuracy of this procedure. The nineteen determinations made by him on different amounts of calcium (0.398, 0.796, and 1.194 mg.) show a maximum single error of 7.87 per cent with an average error of 2.4 per cent. Jansen (5) objects to the use of dilute permanganate solutions because the end-point is not definite and because the value of the solution must be redetermined at frequent intervals. The last objection does not apply to properly made permanganate solutions (22) any more than to any very delicate standard solutions—since all need frequent standardization, and the objection might well be extended to the 0.01 N sodium hydroxide and to the 0.01 N sodium thiosulfate which he uses.

TABLE III.

Effects of Various Salts on the Quantitative Determination of Calcium by the Direct Method.

Ca determined in 3 cc. samples.*		Time allowed for precipitation.	Salts added.†		Total volume of fluid from which Ca was precipitated.
Horse 1, Plasma sample IV.					
mg.	*per cent*	*hrs.*	*salts*	*mg.*	*cc.*
0.382	100.0‡	16			4.5
0.391		16			4.5
0.375	96.9	16	$(NH_4)_2SO_4$	300	7.5
0.370		16	$(NH_4)_2SO_4$	300	7.5
0.380		16	$(NH_4)_2SO_4$	300	7.5
0.357	89.6	16	Na_2SO_4§	300	7.5
0.336		16	Na_2SO_4§	300	7.5
Horse 1, Plasma sample V.					
0.356	100.0‡	16			4.5
0.356		16			4.5
0.359		16			4.5
0.360‖	100.5	16			
0.359	100.5	16	NH_4Cl	300	7.5
0.361		16		300	7.5
0.356	100.0	16	NaCl	300	7.5
0.359		16		300	7.5
0.347	91.7	16	NH_4NO_3	300	7.5
0.338	94.1	16	$(NH_4)_2SO_4$	300	7.5
0.336					
Horse 1, Plasma sample VI.					
0.356	100.0‡	17			6
0.352		20			6
0.356		20			6
0.349‖	98.3	20			
0.338	95.2	0.5			6
0.343	97.7	1.4			6
0.350		1.4			6
0.321	90.4	0.25	NH_4Cl	30.0	
0.314	85.0	0.5	NH_4Cl	30.0	9
0.290		0.5	NH_4Cl	30.0	9
0.329	92.7	1.3	NH_4Cl	30.0	9
0.354	99.7	17.0	NH_4Cl	30.0	9

* Calcium precipitated by Halverson-Bergeim method (10).

† Salts added in the form of 10 per cent calcium-free solutions, except in samples of Horse 1, Plasma VI, where 1 per cent ammonium chloride was added.

‡ By assumption, for purpose of comparison.

§ The anhydrous salt.

‖ Samples ashed in platinum, residues dissolved in hot (6 N) hydrochloric acid, evaporated to 1 to 2 cc., and calcium precipitated by Halverson-Bergeim method (10).

TABLE III—*Continued.*

Ca determined in 3 cc. samples.*		Time allowed for precipitation.	Salts added.†		Total volume of fluid from which Ca was precipitated.
Horse 1, Plasma sample VI—Continued.					
mg.	*per cent*	*hrs.*	*salts*	*mg.*	*cc.*
0.354	98.6	20	NH_4Cl	30.0	9
0.345		20	NH_4Cl	30.0	9
0.323	90.9	0.25	NH_4Cl	30.0	16
0.347	97.7	17.0	NH_4Cl	30.0	16
Dog 3, Plasma sample I.					
		16			4.5
0.255	100.0‡	16			4.5
0.262		16			4.5
0.269		16			4.5
0.265		16	NH_4Cl	300	7.5
0.269	99.2	16	NH_4Cl	300	
0.253		16	NH_4Cl	600	10.5
0.272	102.2	16	NH_4Cl	600	10.5
0.267		16	$MgSO_4$¶	9	7.5
0.269	100.4	16		9	7.5
0.258		16	$MgSO_4$	9	8.5
0.267	102.2		NH_4Cl	100	
0.272		16	$MgSO_4$	9	8.5
			NH_4Cl	100	
Dog 3, Plasma sample II.					
0.258	100.0‡	29			4.5
0.272		29			4.5
0.268‖	101.1	29			
0.166	62.6	1	NH_4Cl	300	7.5
0.173	65.3	2	NH_4Cl	300	7.5
0.270	101.9	29	NH_4Cl	300	7.5
0.120	45.3	1	$(NH_4)_2SO_4$	300	7.5
0.170	64.1	2	$(NH_4)_2SO_4$	300	7.5
0.235	88.7	29	$(NH_4)_2SO_4$	300	7.5
Calf 1, Plasma sample III.					
0.317	100.0‡	16			4.5
0.308		16			4.5
0.306	99.0	16	NH_4Cl	300	7.5
0.313		16	NH_4Cl	300	7.5

¶ All magnesium sulfate expressed as anhydrous salt.

TABLE III—*Concluded.*

Ca determined in 3 cc. samples.*		Time allowed for precipitation.	Salts added.†		Total volume of fluid from which Ca was precipitated.
Calf 1, Plasma sample III—Continued.					
mg.	*per cent*	*hrs.*	*salts*	*mg.*	*cc.*
0.235	75.1	1	NH_4Cl	300	7.5
0.272	86.9	3	NH_4Cl	300	7.5
0.207	66.1	1	$(NH_4)_2SO_4$	300	7.5
0.272	86.9	3	$(NH_4)_2SO_4$	300	7.5
0.313	100.0	16	$(NH_4)_2SO_4$	300	7.5
Calf 1, Plasma sample IV.					
					4.5
0.304	100.0‡	16			4.5
0.315		16	$MgSO_4$	3.0	5.5
0.304	99.3	16	$MgSO_4$	3.0	5.5
0.308		16	$MgSO_4$	6.0	6.5
0.304	99.3	16	$MgSO_4$	6.0	6.5
0.308		16	$MgSO_4$	6.0	7.0
0.306	98.4	16	NH_4Cl	50.0	
0.304		16	$MgSO_4$	6.0	7.0
			NH_4Cl	50.0	

The following experiments were made in order to establish more completely the accuracy of estimating small amounts of oxalate by titration with 0.01 N permanganate:

Calcium oxalate was prepared from best grade reagents ($CaCO_3$, HCl, NH_4OH, $H_2C_2O_4$ and the purity of the salt determined by converting 1 gm. duplicates into calcium sulfate (in platinum) and weighing as such. (Tests for Cl and CO_2 were negative.) Solutions of different calcium concentration were then prepared by dissolving the calcium oxalate in approximately N sulfuric acid. The exact calcium content of each solution was determined as follows: triplicate samples were measured (into platinum dishes) from a calibrated burette, evaporated on a water bath (carefully protected from dust), dried at 110°C., ignited gently, moistened with sulfuric acid, heated to bright redness, and weighed. The treatment with sulfuric acid and heat was repeated until a constant weight was obtained. Samples for titration, in which the amount of calcium varied from 0.02 to 0.596 mg., were measured from a 10 cc. burette (calibrated in 0.02 cc.). All titrations were made in a volume of 5 cc. of approximately normal sulfuric acid at an initial temperature of 75°C.

TABLE IV.

Time Required for the Complete Precipitation of Calcium in the Plasma by the Direct Method.

Ca determined.		Time allowed for precipitation.	Total volume of fluid from which Ca was precipitated.
Horse 2, Plasma sample I.*			
mg.	*per cent*	*hrs.*	*cc.*
0.560	95.7	2	28.5
0.574		2	28.5
0.562	95.7	Less than 4.	23.0
0.565		Less than 4.	23.0
0.574		Less than 4.	23.0
0.589	100.0†	16	23.0
0.594		11	23.0
Calf 1, Plasma sample III.†			
0.272	86.2	1	4.5
0.267		1	4.5
0.283	93.0	3	4.5
0.297		3	4.5
0.317	100.0‡	16	4.5
0.308		16	4.5
Dog 3, Plasma sample II.†			
0.166	62.6	1	4.5
0.170		1	4.5
0.189	69.8	2	4.5
0.191		2	4.5
0.231	83.7	3	4.5
0.212		3	4.5
0.258	100.0‡	29	4.5
0.272		29	4.5
0.268§		29	

* 5 cc. samples of this plasma were diluted with 15 cc. of distilled water previous to the addition of 10 cc. of 1 per cent ammonium oxalate.

† 3 cc. samples used for calcium determinations, 1.5 cc. of 3 per cent ammonium oxalate added to each.

‡ By assumption, for purpose of comparison.

§ Sample ashed in platinum, etc., calcium precipitated by Halverson-Bergeim procedure (10).

The results of these determinations (a total of 193) are given in Table V. From these data, which, with the exception of about a dozen lost in handling, include all samples measured out, it is evident that the average error[22] is approximately 2 per cent.

TABLE V.

*Micro Determination of Calcium (in Pure Solution) by Titration of the Oxalate with 0.01 Normal Potassium Permanganate.**

No. of samples.	Theoretical amounts of Ca taken for determination.		Error of titration.		Manner in which the Ca determined varied from the theoretical amount.		
	Minimum.	Maximum.	Maximum for any single sample.†	Mean for total number of samples.†			
Solution 1.‡ Gravimetric determinations; 0.276; 0.277; 0.275 mg. of Ca per cc.							
	mg.	*mg.*	*per cent*	*per cent*	(−)	(+)	(±)
76	0.028	0.596	+5.4	2.06	45	20	11
Solution 2.‡ Gravimetric determinations; 0.207; 0.206; 0.207 mg. of Ca per cc.							
86	0.021	0.369	−6.0	1.91	61	16	9
Solution 3.‡ Gravimetric determinations; 0.231; 0.232 mg. of Ca per cc.							
17	0.069	0.116	+4.3	1.71	8	4	5
Solution 4.‡ Gravimetric determinations; 0.180; 0.177; 0.182 mg. of Ca per cc.							
14	0.056	0.180	+4.2	1.94	5	9	2
Total of 193 determinations with mean error of 1.91 per cent.							

* Titrations made in a solution of approximately normal sulfuric acid and at an initial temperature of 75° C.

† Calculations made with the assumption that the amount of calcium oxalate measured out was 100 per cent accurate.

‡ Solutions prepared by dissolving $CaC_2O_4 \cdot H_2O$ in approximately normal sulfuric acid. Exact calcium values determined as described in the * to Table VI.

[22] As a basis for calculation it is assumed that the measured amounts of calcium oxalate are 100 per cent accurate.

The numerous determinations of calcium made on pure solutions and on solutions of "artificial blood salts" (*i.e.*, solutions containing phosphate, bicarbonate, chloride, sulfate, sodium, potas-

TABLE VI.

*Recovery of Calcium from Pure Solutions.**

Ca present.	Ca determined.†	Amount of Ca lost or gained in the procedure.		Remarks.
mg.	*mg.*	*mg.*	*per cent*	
0.110	0.111	+0.001	+0.9	Methyl red used as an indicator, pH of solutions approximately 5.6.
0.110	0.104	−0.006	−5.0	
0.338	0.329	−0.009	−2.7	
0.338	0.337	−0.001	−0.3	
0.040	0.037	−0.003	−7.5	
0.040	0.040	±0.000	±0.0	
0.040	0.037	−0.003	−7.5	
0.360	0.363	+0.003	+0.9	
0.110	0.106	−0.004	−3.7	Phenol red used as an indicator, pH of solutions approximately 6.5.‡
0.110	0.106	−0.004	−3.7	
0.110	0.104	−0.006	−5.0	
0.338	0.339	+0.001	+0.3	
0.360	0.361	+0.001	+0.3	
0.360	0.364	+0.004	+1.1	
0.360	0.364	+0.004	+1.1	
0.360	0.351	−0.009	−2.5	
0.360	0.348	−0.012	−3.3	
0.360	0.352	−0.008	−2.1	
0.360	0.364	+0.004	+1.1	
0.360	0.355	−0.005	−1.4	

* Solutions prepared by converting pure $CaC_2O_4 \cdot H_2O$ into CaO, dissolving the CaO in dilute HCl, and diluting to definite volume. The exact calcium values were determined by evaporation of relatively large volumes (25 cc.), conversion to $CaSO_4$, and weighing as such. All analytical work carried out in platinum dishes.

† Calcium precipitated according to the Halverson-Bergeim procedure (10).

‡ Small amounts of normal NH_4OH added to obtain this pH. With the prescribed amounts of sodium acetate (10, 23) the pH varies from 5.2 to 5.6.

sium, magnesium, etc., in concentration approximately the same as found in circulating blood), as well as the procedures of the various micro methods are based largely on the work of Richards,

TABLE VII.

Determination of Calcium in Solutions of "Artificial Blood Salts."

Ca present.*	Ca determined.†	Amount of Ca lost or gained in the procedure.		Salts present in solution.		Remarks.
mg.	*mg.*	*mg.*	*per cent*	*salts*	*mg.*	
0.110	0.113	+0.003	+2.7	Na_2HPO_4‡ KH_2PO_4 NaCl Na_2CO_3§	1.6 0.6 1.7 9.0	Phenol red used as indicator, pH approximately 6.5.¶
0.360	0.360	0.000	0.0	Na_2HPO_4 KH_2PO_4 NaCl Na_2CO_3	0.8 0.3 0.8 4.5	"
0.360	0.360	0.000	0.0	As above.	As above.	"
0.360	0.365	+0.005	+1.4	Na_2HPO_4 KH_2PO_4 NaCl Na_2CO_3	3.2 1.2 3.5 18.0	"
0.360	0.369	+0.009	+2.7	Na_2HPO_4 KH_2PO_4 NaCl Na_2CO_3 $MgSO_4$‖	3.2 1.2 3.5 18.0 15.0	"
0.360	0.362	+0.002	+0.5	As above.	As above.	"
0.360	0.381	+0.021	+5.8	" "	" "	Phenol red used as indicator, pH 7.2 using ammonium hydroxide in place of sodium acetate.
0.360	0.381	+0.021	+5.8	" "	" "	"

* Same solutions used as described by * in Table VI.

† Calcium precipitated by the Halverson-Bergeim procedure (10).

‡ $Na_2HPO_4 \cdot 12H_2O$.

§ Anhydrous Na_2CO_3.

‖ Calculated as anhydrous $MgSO_4$.

¶ Normal ammonium hydroxide used in place of sodium acetate.

TABLE VIII.

Recovery of Added Calcium from Whole Blood and Plasma by the Method of Direct Precipitation.

Ca present* (in plasma or blood).	Ca added (as $CaCl_2$).	Total Ca.		Amount of added Ca recovered.†	
		Present.†	Determined.		
Horse 1, Plasma sample II A.					
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
0.500					
0.496					
Average......0.498	0.266	0.764	0.782	0.284	106.7
Horse 1, Plasma sample II B.					
0.560					
0.554					
	0.213	0.770	0.775	0.218	102.3
	0.319	0.876	0.880	0.323	101.2
Average......0.557	0.266	0.823	0.833	0.276	104.5
Horse 1, Plasma sample III A.					
0.553					
0.553					
	0.106	0.659	0.663	0.110	103.8
Average......0.553	0.213	0.766	0.780	0.227	106.6
Horse 1, Plasma sample III B.					
0.116‡					
0.118‡					
Average......0.117	0.106	0.223	0.222	0.105	99.1
Horse 1, Whole blood sample III.					
0.275§					
0.262§					
0.264§					
Average......0.267	0.096	0.363	0.359	0.092	95.8

* Except where otherwise noted, 5 cc. samples were used.

† It is assumed that the calcium measured out was 100 per cent accurate. This amount plus the average of the control determinations make up the total amount present.

‡ 1 cc. samples of plasma.

§ Although the original samples contain 5 cc. of whole blood, the sample from which the Ca is determined is only 3 cc., and the 0.096 mg. of Ca represents only a corresponding part (0.6) of the added calcium.

TABLE VIII—*Concluded.*

Ca present* (in plasma or blood).	Ca added (as $CaCl_2$).	Total Ca. Present.†	Total Ca. Determined.	Amount of added Ca recovered.†	
Horse 2, Plasma sample III.					
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
0.600					
0.605					
	0.183	0.786	0.782	0.179	97.8
	0.121	0.724	0.717	0.114	94.2
	0.154	0.757	0.765	0.162	105.2
Average......0.603	0.202	0.805	0.812	0.209	103.4
Horse 2, Plasma sample IV.					
0.617					
0.620					
0.617					
0.615					
	0.082	0.699	0.705	0.088	107.3
	0.106	0.723	0.724	0.107	100.9
	0.163	0.780	0.790	0.173	106.1
	0.139	0.756	0.761	0.144	103.7
Average......0.617	0.065	0.682	0.680	0.063	97.0
Beef plasma, Sample I.					
0.513					
0.509					
0.514					
0.518					
	0.152	0.664	0.667	0.155	102.0
	0.110	0.622	0.617	0.105	95.5
Average......0.512	0.136	0.648	0.646	0.134	98.5
Beef serum, Sample I.					
0.638					
0.636					
0.645					
	0.360	1.000	1.011	0.371	103.0
Average......0.640	0.360	1.000	1.011	0.371	103.0

TABLE IX.

Comparison of Calcium Values Obtained by Various Methods.

Amount of blood or plasma taken. Original sample.	Amount of blood or plasma taken. Aliquot used in actual determination of Ca.	Ca actually determined in sample.	Ca estimated per 100 cc.	Methods used.
				Calf 1, whole blood.
cc.	*cc.*	*mg.*	*mg.*	
5	3	0.270	8.97	Direct precipitation.*
5	2.1	0.191	8.92	Deproteinization with picric acid.†
5	2.1	0.177	8.28	" " " "
5	2.5	0.212	8.46	" " trichloroacetic acid.‡
5	2.5	0.205	8.19	" " " "
5	2.5	0.048	1.93§	" " tungstic acid.‖
5	2.5	0.039	1.56§	" " " "
				Calf 1, plasma.
3	3	0.327	10.89	Direct precipitation.*
3	3	0.329	10.97	" "
5	2	0.200	10.00	Deproteinization with picric acid.†
5	2	0.190	9.50	" " " "
5	5	0.566	11.32	Ashed in platinum.¶
				Dog 1, whole blood.
5	2.5	0.168	6.72	Deproteinization with trichloroacetic acid.**
5	2.5	0.170	6.81	" " " "
5	2.5	0.147	5.89	" " " " ‡
5	2.5	0.143	5.70	" " " "
5	5.0	0.258	5.15	Ashed in platinum.¶
5	3	0.168	5.60	Direct precipitation.*
5	3	0.157	5.23	" "
5	3	0.163	5.44	" "
5	3	0.166	5.52	" "

* Method as published in this paper.

† Regular procedure of Halverson and Bergeim (10).

‡ 5 cc. samples of plasma or whole blood diluted with 25 cc. of distilled water. 15 cc. of 6.5 per cent trichloroacetic acid are then added and after making up to volume (50 cc.) the mixture is well shaken. Calcium determined in aliquot by Halverson-Bergeim method (10).

§ Precipitated mixtures heated in boiling water for 3 minutes before filtering.

‖ Deproteinization with tungstic acid (17), calcium precipitated by Halverson-Bergeim procedure (10).

¶ Samples ashed in platinum, residues dissolved in hot (6 N) hydrochloric acid, evaporated to small volume (1 to 2 cc.), and calcium precipitated by Halverson-Bergeim method (10).

** Deproteinization by Lyman's procedure (9), calcium precipitated by Halverson-Bergeim procedure (10).

TABLE IX—*Continued.*

Amount of blood or plasma taken. Original sample.	Amount of blood or plasma taken. Aliquot used in actual determination of Ca.	Ca actually determined in sample.	Ca estimated per 100 cc.	Methods used.
				Dog 1, plasma.
cc.	*cc.*	*mg.*	*mg.*	
5	5	0.449	8.97	Ashed in platinum.¶
3	3	0.267	8.90	Direct precipitation.*
3	3	0.262	8.73	" "
3	3	0.269	8.97	" "
				Dog 2, Plasma sample I.
3	3	0.258	8.59	Direct precipitation.*
3	3	0.260	8.66	" "
3	3	0.262	8.74	" "
3	3	0.253	8.43	" "
5	5	0.422	8.79	Ashed in platinum.¶
5	2.7	0.258	9.57	Deproteinization with picric acid.†
5	2.0	0.175	8.74	" " trichloroacetic acid.‡
				Dog 2, Plasma sample II.
5	2.7+	0.248	9.17	Deproteinization with picric acid.†
5	2.7	0.230	8.53	" " " "
5	2.5	0.204	8.17	" " trichloroacetic acid.‡
5	2.5	0.222	8.86	" " " "
5	2.0	0.107	5.34	" " tungstic acid.‖
5	2.0	0.123	6.16	" " " "
5	5.0	0.502	10.03	Ashing in platinum.¶
3	3.0	0.297	9.89	Direct precipitation.*
3	3.0	0.308	10.27	" "
				Dog 3, Plasma.
3	3	0.255	8.51	Direct precipitation.*
3	3	0.262	8.74	" "
3	3	0.269	8.97	" "
3	3	0.265	8.82	" "
3	3	0.258	8.59	" "
5	5	0.446	8.92	Ashing in platinum.¶
5	2	0.195	9.75	Deproteinization with trichloroacetic acid.**
5	2.5	0.224	8.97	" " " " ‡

TABLE IX—*Concluded.*

Amount of blood or plasma taken. Original sample.	Amount of blood or plasma taken. Aliquot used in actual determination of Ca.	Ca actually determined in sample.	Ca estimated per 100 cc.	Methods used.
				Horse 1, Plasma sample II.
cc.	*cc.*	*mg.*	*mg.*	
5	2.5	0.190	7.60	Deproteinization with tungstic acid.‖
5	2.5	0.200	8.00	" " " "
5	2.5	0.281	11.24	" " picric acid.†
5	2.5	0.269	10.76	" " " "
5	5.0	0.570	11.40	Ashing in platinum.¶
5	5.0	0.568	11.36	" " "
				Horse 1, Whole blood sample IV.
5	2.1	0.253	11.82	Deproteinization with picric acid.†
5	2.1	0.242	11.32	" " " "
5	2.1	0.237	11.07††	" " " "
5	2.1	0.230	10.79††	" " " "
5	2.5	0.097	3.86§	" " tungstic acid.‖
5	2.5	0.085	3.40§	" " " "
5	2.5	0.244	9.74	" " trichloroacetic acid.‡
5	2.5	0.238	9.51	" " " "
5	3.0	0.287	9.58	Direct precipitation.*
5	3.0	0.290	9.66	" "
5	3.0	0.301	10.04	" "
5	3.0	0.279	9.30	" "
5	5.0	0.456	9.11	Ashing in platinum.¶
				Horse 1, plasma.
3	3	0.370	12.34	Direct precipitation.*
3	3	0.372	12.42	" "
3	3	0.368	12.27	" "
5	1.4	0.179	12.54	Deproteinization with picric acid.†
5	2.1	0.266	12.43	" " " "
5	2.5	0.216	8.65	" " tungstic acid.‖
5	1.5	0.113	7.51	" " " "
5	2.5	0.223	8.92‡‡	" " " "
5	1.5	0.131	8.74‡‡	" " " "
5	5.0	0.617	12.33	Ashing in platinum.¶

†† Precipitation made at room temperature.

‡‡ Precipitated mixture heated in boiling water for 5 to 6 minutes previous to filtering.

McCaffrey, and Bisbee (30) and especially upon that of McCrudden (24). While checking over the different methods, a number of control determinations were made[15] on pure calcium solutions and on solutions of artificial blood salts. The results, given in Tables VI and VII, show that even with very small amounts of calcium (not over 0.38 mg.) the average error[18] of recovery (2.4 per cent) differs but little from the error found in the direct titration of small amounts of calcium oxalate (see Table V). Brief mention should be made of the fact that the errors found in the titration of pure calcium oxalate and in the determination of calcium in the various known solutions cannot be wholly attributed to the dilute permanganate, for in measuring out samples as small as 0.1 cc. a difference of 0.005 cc. would cause an error of 0.5 per cent.

Looking over the numerous micro methods which use potassium permanganate (3, 6–10, 12–15), one finds considerable variation in the conditions under which various investigators have made oxalate titrations. Considering only the oxidation of oxalates by permanganate (for a complete discussion of all phases of permanganate reactions consult the papers of Harcourt and Esson (33), Ehrenfeld (34), Luther and Schilow (35), Schilow (36), Schroeder (37), Skrabal (38), Sarkar and Dutta (39), it has been shown by Bray (40) and McBride (41) that the results obtained depend upon: (1) concentration of H ion; (2) temperature; (3) rate of addition of the permanganate; and (4) dilution.

It was found by McBride (41) that, with 0.1 N potassium permanganate, the optimum acidity of oxalate solutions was 5 per cent, amounts less than this decreased the amount of premanganate necessary for the end-point while higher concentrations of acid led to an evolution of oxygen and required additional permanganate for the end-point. Low temperatures led to a decreased rate in reduction, a conditon favorable to the accumulation of $HMnO_4$ or MnO_2 with subsequent evolution of oxygen. The rate of addition is most important in the beginning of the reaction; if a large excess of permanganate is present before rapid oxidation of the oxalate is in progress or if insufficiently stirred at any step, the conditions are again favorable for the evolution of oxygen. A summary of the conditions necessary for accurate titration of oxalate by permanganate would be:

A dilute solution, acid concentration 5 per cent, initial temperature of 70–80°C., slow addition of permanganate at beginning, solution to be thoroughly stirred at all times and the final temperature not lower than 60°C. The writer titrated a number of samples of pure calcium oxalate with 0.01 N permanganate, using 1, 1.5, and 3 N sulfuric acid and at temperatures varying from 60–85°C. The results with the 0.01 N permanganate agreed with those obtained by McBride (41) with the 0.1 N; at temperatures above 80°C. and with acid stronger than 1.5 N, the end-point is very indefinite and too much permanganate is used. (Halverson and Bergeim (10) report similar results with high acidity and high temperatures.) With initial temperatures as low as 60°C. the rate of reaction is very slow and the end-point is extremely variable. The writer found that in the micro-titrations (5 cc. of acid in a 50 cc. centrifuge tube) the temperature dropped from 17–25°C. during the short time required (1 to 1.5 minutes) for the titration, so if the initial temperature were only 60°C. the end-point would be reached at about 40°C., which has been found (40) to be undesirable. It is therefore apparent that the indefinite temperatures of Krüger (32), Lamers (3), and Kramer and Howland (8) as well as the low temperatures used by Halverson and Bergeim (10), de Waard (7), Dienes (6), and Kramer and Tisdall (14) are not compatible with the best results.

The accuracy of the direct method has been checked by the following experiments:

(1) By addition of known amounts of calcium and subsequent recovery. The results of this work, presented in Table VIII, show that the average error[23] of recovery from plasma is approximately 3.7 per cent. (2) By a comparison of calcium values obtained by different methods on several large samples of whole blood and plasma. The results of these analyses, given in Table IX, show that the calcium values obtained by the direct method from whole blood and plasma, while differing but slightly from those obtained after ashing in platinum (the standard for comparison), are lower than those obtained by any of the deproteinization methods.

SUMMARY.

1. Experimental data have been presented to show that the micro method depending on the direct precipitation of calcium from serum, plasma, and whole blood is accurate to ± 5 per cent, and equal in this respect to any of the numerous micro methods found in the literature.

2. The direct method possesses several advantages not common to other procedures: (1) The small amount of sample necessary (1

[23] It is assumed that the measured amounts of calcium chloride are 100 per cent accurate.

to 5 cc. of plasma, 5 cc. of whole blood); (2) the saving in time (no ashing, protein precipitation, filtering, etc.); and (3) minimal mechanical loss, since all operations are carried out in one tube.

3. With proper concentration of H ions (approximately that of N sulfuric acid) and at an initial temperature of 75° C., small amounts of calcium may be accurately estimated (to approximately 2 per cent) by titration of the oxalate with 0.01 normal potassium permanganate.

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THE FATE OF SULFIDES IN THE BLOOD.*

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The investigation embodied in this paper was primarily undertaken in an endeavor to determine in what manner H_2S is transported in the blood after inhalation of this gas. That it is transported in the blood in a loosely combined and readily dissociable manner is evident from the fact that, when H_2S is injected into any of the body cavities, it is almost at once detectable in the expired air (1). On the question in what form hydrogen sulfide combines with and is carried by the blood there is diversity of opinion, however.

Two modes of transportation have been suggested: That the hydrogen sulfide combines with the hemoglobin to form a sulfhemoglobin compound; and that the gas in its capacity as an acid unites with the alkali of the blood to form sodium sulfide. Since both of these combinations are dissociable both views necessarily assume a trace of H_2S in simple solution in the plasma.

Paradoxically, although the first of these views is definitely disproved by evidence contained in the literature, the belief that inhalation of H_2S is followed by the formation of a sulfur hemoglobin compound is still generally prevalent. The persistence of this idea is no doubt due in part at least to the need for an explanation of the rare disease, sulfhemoglobinemia, one of the endogenous cyanoses.

* This is one of a series of papers dealing with hydrogen sulfide poisoning. Other papers of this series will probably appear in the American Journal of Physiology, the Journal of Pharmacology and Experimental Therapeutics, and the Journal of Industrial Hygiene.

The reaction of hemoglobin to H_2S has been the subject of much study. Hoppe-Seyler (2) in 1863 and later Araki (3), by passing a stream of H_2S through oxygenated blood, obtained a dirty greenish pigment. It showed an absorption spectrum with two bands in the red, one quite close to C and the other midway between C and D. This compound was designated sulfmethemoglobin. The green discoloration seen in cadaveric decomposition was assigned to the postmortem formation of sulfmethemoglobin.

Gamgee (4) in 1898 branded the sulfmethemoglobin of Hoppe-Seyler and Araki as a mixture of decomposition products. Harnack (5), a year later, while also emphatically denying the existence of this substance, obtained in solution by the action of H_2S upon reduced hemoglobin, a compound which he designated as sulfhemoglobin. Its absorption spectrum gave but a single band between the Fraunhofer lines C and D and extending from $\lambda.610$ to $\lambda.625$.

In 1907, Clarke and West (6) verified Harnack's work and attempted to isolate the compound, but were unsuccessful. They noted that complete reduction of the hemoglobin was essential to the formation of this substance. They found further, that very high concentrations of H_2S were necessary to force a union between hemoglobin and this gas. These concentrations were far higher than those which would be instantly fatal if inspired. The presence of powerful reducing agents, such as phenylhydrazine, however, greatly facilitated the reaction and rendered a partial combination possible at relatively low concentrations of H_2S and even in the presence of oxyhemoglobin.

It is on the basis of this last observation that the occurrence, during life, of blood giving the spectrum of sulfhemoglobin—the characteristic of the disease known as sulfhemoglobinemia—is explicable. Wallis (7) found that the formation of sulfhemoglobin in the blood of patients suffering from this disease, is due to the presence in their blood of a strong reducing agent, a hydroxylamine derivative, which presumably comes from the splitting of protein by a nitrosobacillus which inhabits the buccal cavities. A mere trace of H_2S is necessary under these conditions to form sulfhemoglobin and this might be derived from the intestinal tract.

From these facts it is apparent that the formation of sufhemoglobin within the living body is primarily dependent upon an abnormal condition of the blood, rather than upon any function of the hemoglobin as the transporting agent of H_2S. Within the body of a healthy living individual—one whose mouth does not contain this nitrosobacillus—complete reduction of the blood would be essential before any combination of the gas with hemoglobin could occur.

As a postmortem change, however, sulfhemoglobin may be rapidly formed through bacterial action. This change, we may presume, consists first in a reduction of the blood and then a combination of the hemoglobin with the H_2S liberated during the process of decomposition.

It is a highly significant fact, that the blood taken very soon after death from H_2S poisoning does not show the spectrum characteristic of sulfhemoglobin and is abnormal, if at all, only in the degree of its reduction (7, 8). *A fortiori* hemoglobin is not the normal transporter of H_2S.

Diakonow (9) was apparently the first to point out that, through its properties as an acid, H_2S should act upon the bicarbonates of the blood plasma to form sodium sulfide. He demonstrated this experimentally upon bicarbonate solutions. Following this lead Pohl (10) came to the conclusion that H_2S must be transported in the blood in this form alone. In support of this view he pointed out the remarkable similarity between poisoning with H_2S gas and that induced by injection of sodium sulfide.

It seems plausible that, to some extent at least, sodium sulfide would be formed within the plasma after inhalation of this gas. For this reason the following experiments were undertaken. In the main, however, they have led to quite another conclusion.

EXPERIMENTAL.

The following experiments which were repeatedly performed, show a marked difference between the reactions toward H_2S of sodium bicarbonate solution on the one hand and of plasma on the other. The former combines with the gas to some extent, presumably as Na_2S and even when washed free of dissolved H_2S gives a persistent test for sulfide. The plasma, however, retains

no sulfide, detectable as such, in any form other than the dissolved H_2S gas. Evidently plasma after exposure to a moderate amount of H_2S does not contain Na_2S.

Experiment 1. The Action of H_2S upon Sodium Bicarbonate Solution.—5 cc. of 0.2 per cent sodium bicarbonate solution were shaken in a flask containing an atmosphere of 0.5 per cent H_2S in air. The flask was then opened and air passed through until the odor of H_2S was no longer detected. The solution was then tested, and gave a sulfide reaction both with lead acetate and with ammoniacal sodium nitroprusside. The passage of pure air, 5.5 per cent CO_2 in air, or oxygen through the liquid for 3 hours did not render it incapable of giving a sulfide test. The addition of dilute HCl to the fluid was attended with the evolution of H_2S.

Experiment 2. The Action of H_2S upon Plasma.—Dog's plasma was shaken in a flask with 0.5 per cent H_2S and then aerated. The plasma failed to give any of the above tests for sulfide nor was H_2S evolved upon the addition of dilute HCl.

This experiment with plasma (Experiment 2) affords a clear-cut and decisive negative on the question to which it is primarily directed. The striking difference from the result with bicarbonate solution (Experiment 1) prompts the further question as to just what does happen when H_2S is brought into contact with plasma.

To investigate this question, further experiments were therefore performed. Plasma was exposed to a definite volume of H_2S in air, nitrogen, CO_2, or oxygen and then washed free of dissolved H_2S with the same atmosphere. The hydrogen sulfide recoverable from the gas was estimated quantitatively. In some cases the CO_2-combining power of the plasma before and after treatment with H_2S was determined as a means of following any change in the sodium bicarbonate of the plasma.

Experiment 3. The Reaction between H_2S and Plasma or Blood.—The CO_2-combining power of a sample of normal plasma from dog's blood was determined at 40 mm. partial pressure CO_2. Some of the plasma was then evacuated of gas by means of a suction pump, and 5 cc. samples were pipetted into 1 liter flasks containing, in successive tests, atmospheres of air, oxygen, nitrogen, and 40 mm. CO_2 in air. To all of these atmospheres, 0.5 per cent H_2S had been added. Each flask was rotated for 1 minute. The H_2S which could be recovered was then determined by aerating the flask with the same atmosphere with which it was filled, minus the H_2S, and drawing the gas through a bead tower containing 0.01 N iodine (11).

In each case at the end of the aeration the plasma was tested for sulfide; but only negative results were obtained.

Samples of whole blood were treated in the same manner except that the initial evacuation was omitted. Table I embodies the results obtained.

From Experiment 3 it appears that not only does H_2S fail to form sodium sulfide when acting upon blood or plasma but that a portion of the gas is actually destroyed. The destruction of

TABLE I.

Atmosphere in flask.	CO_2-combining power of plasma at 40 mm. partial pressure. Before exposure.	After exposure.	H_2S recovered.	H_2S lost.	H_2S oxidized.
Reaction between plasma and H_2S.					
	vol. per cent	*vol. per cent*	*cc.*	*cc.*	*per cent*
Air and 5.2 cc. of H_2S	54	40	3.10	2.10	40
" " 4.6 " " H_2S	54	46	2.94	1.66	36
" " 5.4 " " H_2S	54	41	3.32	2.08	38
" with CO_2 at 40 mm. and 5.7 cc. of H_2S	54	45	3.42	2.28	40
" " CO_2 " 40 " " 5.5 " " H_2S	54	44	3.70	1.8	34
Oxygen and 5.6 cc. of H_2S			2.11	4.49	80
" " 5.4 " " H_2S			2.4	4.0	74
Nitrogen and 5.0 cc. of H_2S			4.60	0.40	8
" " 5.1 " " H_2S			4.42	0.68	13
Reaction between whole blood and H_2S.					
Air and 5.4 cc. of H_2S			1.81	3.59	66
" " 5.1 " " H_2S			1.96	3.14	62
" " 5.4 " " H_2S			2.00	3.40	60

H_2S is here seen to be dependent upon the presence of oxygen and is, therefore, an oxidation. The oxidized products combine in part with the alkali of the plasma and decrease its CO_2-combining power.

Obviously this raises the question whether the loss in alkali, as measured by the CO_2-combining power of the plasma, is adequate to account for the amount of H_2S destroyed on the basis of a bivalent combination of sulfur (H_2SO_4) with the sodium. Cal-

culation shows, however, that the oxidation of 1 cc. of H_2S would produce enough sulfuric or other bivalent acids to reduce the CO_2 capacity of 5 cc. of blood or plasma by 20 volumes per cent. The figures actually obtained are only 20 to 30 per cent of this amount. A portion of the oxidation products must, therefore, be in combination with constituents of the blood other than alkali, or otherwise concealed.

In oxygenated whole blood the oxidation of hydrogen sulfide is more complete than in plasma. This is due apparently not to a catalytic action of the corpuscles, but to the greater available supply of oxygen. The withdrawal of oxygen from the corpuscles in the oxidation of hydrogen sulfide results in the deoxygenation of the blood. This process affords the explanation of the reduction of hemoglobin by H_2S, which has been observed by previous workers (6).

Not only does the plasma fail to combine H_2S with alkali to form sodium sulfide but, as will be seen in Experiment 4, plasma has the property of dissociating sodium sulfide and liberating H_2S from it. The absence of oxygen has no inhibitory influence upon this reaction. In the presence of oxygen, however, a portion of the liberated H_2S is oxidized. The processes of dissociation and oxidation are to some extent distinct and independent each from the other. The first is apparently of the nature of a catalytic acceleration of the well recognized and easily demonstrated reaction of sodium sufide with water:

$$Na_2S + 2H_2O = H_2S + 2NaOH.$$

The oxidation on the other hand possesses many of the characteristics of oxidative reactions which occur through the action of tissue juices. The importance of the part played by the SH group in vital oxidations is beginning to attract particular attention (12).

The whole phenomenon of hydrolysis and oxidation can be illustrated quite simply by an experiment designed by Clarke and West (6) to demonstrate the reduction of blood by sodium sulfide. To the bottom of a test-tube of blood is passed a small amount of a strong solution of sodium sulfide. Gradually the area above the sulfide becomes reduced and this reduction progresses up the tube. It is a highly significant fact that even before the

upper layer changes color the odor of the liberated and unoxidized H_2S can be detected.

In this or any similar experiment the sodium sulfide added to the blood or plasma becomes completely hydrolyzed, and with adequate aeration the H_2S thus formed is either oxidized or volatilized off. Thereafter the blood or plasma does not respond to tests for sulfides. As would be anticipated, one result of the hydrolysis of the sulfide is that the sodium increases the alkali in the fluid and raises its CO_2-combining power. If the oxidation is active a portion of the liberated sodium is used up in combining with the products of oxidation, presumably in part at least sulfuric acid.

Experiment 4. The Reaction of Sodium Sulfide with Blood and Plasma.—Samples, 5 cc. each, of dog's plasma were evacuated of gas, and then placed in flasks containing atmospheres of air, oxygen, or nitrogen. 3 cc. of approximately 0.8 per cent sodium sulfide solution were added. The aerating gas was then passed through the mixture, and the H_2S carried over was absorbed in 0.01 N iodine and calculated as in the previous experiment. The amount of H_2S recoverable when acidulated water was used instead of blood was 6.1 cc.; this figure, 6.1, has been used throughout as a control. After the plasma had been washed free of H_2S, the fluid was tested for sulfide, with negative results in all cases. The whole blood used was treated in a manner similar to the plasma except that the initial exhaustion was omitted. Table II embodies the results obtained.

TABLE II.

Atmosphere in flask.	H_2S recovered.	H_2S lost.	H_2S oxidized.
The reaction of sodium sulfide with plasma.			
	cc.	*cc.*	*per cent*
Air	2.9	3.2	53
"	2.6	3.5	57
"	3.0	3.1	51
Nitrogen	5.9	0.2	3
"	5.6	0.5	8
Reaction of sodium sulfide with whole blood.			
Air	1.2	4.9	80
"	1.6	4.5	74
Oxygen	1.0	5.1	83

Experiment 4 illustrates the hydrolysis of sodium sulfide by plasma or whole blood and the oxidation of the liberated H_2S in the presence of oxygen.

The hydrolyzation of Na_2S by plasma and the subsequent oxidation of a portion of the liberated H_2S is extremely rapid. This is seen in the following experiment which was designed primarily to determine whether or not the products of oxidation were toxic.

Experiment 5. The Detoxication of Sodium Sulfide by Plasma.—To 10 cc. of dog's plasma was added 0.6 gm. of Na_2S in 10 per cent water solution and the whole shaken for 1 minute with a stream of air from the blower passing through the flask. At the end of this time 1 cc. of the mixture was tested with negative results for the presence of sulfide. The rest of the plasma thus treated was injected intravenously into a 10 kilo dog. The inoculation was entirely without effect upon the animal although the amount of Na_2S added to the plasma was ten times the ordinary lethal dose.

From this experiment we may conclude that the oxidation products of H_2S or sodium sulfide in the blood are non-toxic. Evidently this process of detoxication may be of considerable importance during the putrefaction of sulfur-containing substances in the intestine. Heretofore it has usually been ascribed to the liver.

In consideration of this point it would seem that when H_2S is inhaled it exists only momentarily in the blood as the dissolved and unoxidized gas. Of course, theoretically, an infinitesimal trace of alkali sulfide must be present. The active physiological effects of H_2S are exerted by the gas in solution in the plasma.

In view of the oxidative process above illustrated in the *in vitro* experiments, it is obvious that *in vivo* the gas can exhibit no cumulative action when inhaled. This is well borne out by other experimental findings, for in observations to be reported in detail in a later paper, it has been found that the inhalation of 5 parts of H_2S in 10,000 parts of air for a period of 11 hours produces no general symptoms in dogs, while the inhalation of 10 parts results in death in 15 minutes.

If the effects were cumulative there would not be this wide variation in the effects of such closely related concentrations. The rapid recovery from non-fatal inhalational poisoning by H_2S is a striking confirmation of the lack of cumulative action of this gas and of the necessity for the maintenance in the blood of a toxic concentration in order to produce the characteristic symptoms of poisoning. An animal removed from an atmosphere

of H_2S in a state of deep coma, frequently appears quite normal after the lapse of a few minutes. The same considerations apply to the effects following intravenous injection of a solution of sodium sulfide. The salt is hydrolyzed in the blood and H_2S is at once apparent in the expired air. If the administration is rapid enough to allow a sufficiently high concentration of unoxidized H_2S in the blood, toxic symptoms and death result.

These points are illustrated in Experiment 6 in which, by slow intravenous injection, many times the lethal amount of sodium sulfide was administered. According to the literature the lethal dose of sodium sulfide for dogs is 6 mg. per kilo, and this has been confirmed here.

Experiment 6. The Repeated Administration of Sodium Sulfide.—A 10 kilo dog was subjected to intravenous injection of sodium sulfide solution (0.6 per cent) at the rate of 2.5 cc. per minute. (This is 25 per cent of the lethal dose each minute.) The animal exhibited some restlessness and a slight dyspnea but, after a total injection of 50 cc. during 20 minutes, was apparently none the worse, although five times the lethal amount had been administered.

A rapid injection of 10 cc. of the solution was then made. After a few gasps the animal became rigid and died.

This experiment also throws some light upon the comparatively slight toxic effects induced by the sulfide formed in the intestine from the decomposition of protein or following the administration of sulfur. The sulfide so formed is slowly absorbed, and completely detoxicated in the blood. Large amounts of sulfur may be thus altered and eliminated without the development of any marked physiological effects although, following the ingestion of sulfur, the breath may be foul with H_2S. In this connection it may be mentioned that about 10 per cent of the sulfur taken by mouth is absorbed as sulfide and later eliminated through the urine as sulfates and in organic combination (13). There are, however, a few reported cases of toxic symptoms following the still prevalent administration of sulfur (14).

Since the capacity of the blood to oxidize H_2S is quite adequate to cope with many times the lethal amount, it is necessary to offer some explanation of the intense physiological activity of this gas which when inhaled ranks close to cyanogen in toxicity (14). The laws of mass action offer a tenable explanation of the phe-

nomena. The dissolved H_2S in the blood is a factor in the equilibrium of the reaction of oxidation. The greater the amount of inhaled H_2S the more active will be the oxidation, but there will be also momentarily a higher concentration of H_2S dissolved in the blood and in consequence a greater physiological effect.

Unless death supervenes the concentration of H_2S dissolved in the blood can only be maintained by the continued inhalation of the gas. When the inhalation ceases the H_2S present in the blood is rapidly and completely destroyed. This chemical process corresponds with the prompt recovery of the man or animal on removal from the atmosphere of H_2S.

The assumption that the dissolved H_2S in the blood exists as an equilibrium factor in the oxidative reaction is supported by an experimental observation. When 10 or 15 cc. of H_2S gas (too small an amount to produce any symptoms, whatever) are injected into the peritoneal cavity of a dog, there soon follows the characteristic smell of the gas in the breath of the animal. When the active oxidation of H_2S shown by the blood in Experiment 3 is considered, it is indeed remarkable that a portion of the gas should escape unoxidized from the lungs, after passing through the venous system where there is certainly sufficient oxygen to destroy it, unless this was due to the persistence of the rapidly decreasing factor, concentration of H_2S, in the trend back to equilibrium.

CONCLUSIONS.

When an atmosphere containing H_2S is inhaled no combination of the gas is formed with the hemoglobin of the blood nor is any appreciable amount of sodium sulfide formed in the plasma. The phenomena of the disease of sulfhemoglobinemia have no significance for the normal transport of sulfide.

Blood plasma in the presence of oxygen possesses the property of rapidly oxidizing H_2S. The products of oxidation combine in part with the sodium of the plasma.

Sodium sulfide is rapidly and completely hydrolyzed by blood or plasma. The absence of oxygen has no effect upon this process. If oxygen is present, however, a large part of the liberated H_2S is oxidized.

The reduction of blood by H_2S or Na_2S is the result of the withdrawal of oxygen from the corpuscles for the oxidation of the H_2S.

After inhalation of H_2S or intravenous administration of Na_2S, the sulfide in the blood exists only as dissolved and as yet unoxidized H_2S. The active physiological effects of sulfides are exerted by the H_2S in solution in the blood. During the administration of sulfide in any manner the H_2S in solution in the blood is a factor in the reaction of oxidation.

The rate of oxidation of H_2S in the blood is such that in a comparatively short period many times the lethal amount of sodium sulfide may be administered intravenously to animals without any apparent effect. This explains the comparatively slight toxic properties exhibited by the absorption of sulfides from the intestional tract.

In conclusion I wish to express my sincere thanks to Professor Yandell Henderson for suggestions and criticism.

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THE PREPARATION OF FLEXIBLE COLLODION MEMBRANES.

By JOSEPH M. LOONEY.

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(Received for publication, November 10, 1921.)

Collodion membranes were first utilized for experiments in diffusion by Fick (1) in 1855. Fick realized their advantages but was unable to develop a suitable method of fastening them to holders and so abandoned their use.

Since his paper was published many authors have described various methods of making and applying these membranes of which a fairly complete bibliography may be found in the paper of Bigelow and Gemberling (2).

The numerous attempts which have been made to control the permeability of these membranes have not been attended by any marked success. It was early known that these membranes became more impermeable on drying, and this fact was utilized by Bigelow and Gemberling (2), and Malfitano (3) to control the permeability of their membranes. No attempt was made to standardize the membranes made in this way as to the degree of permeability except to state that the longer the membranes were allowed to dry the less permeable they became. A more serious attempt was made in this direction by Walpole (4), who controlled his membranes by measuring the thickness of the film with a micrometer. He allowed a definite weight of collodion to evaporate on a glass plate of known area to given thickness and thus obtained a definite degree of drying, which could be accurately described by giving the ratio of the weight of the film to its weight when completely dry.

Brown (5) describes a method in which the permeability is varied by treating the dry membranes with different strengths of ethyl alcohol. He dips a small test-tube into an 8 per cent solution of collodion in a mixture of equal parts of absolute ethyl

alcohol and ether, and allows the membrane to drain for 5 minutes while held in a vertical position in an inverted Erlenmeyer flask. The tube is immediately immersed in water for about 1 minute, and the hardened collodion film is then stripped off. The membrane thus obtained is allowed to dry over night at room temperature, which renders it highly impermeable. When these membranes are soaked for 24 hours in different strengths of alcohol their permeability increases correspondingly to the increase in the concentration of the alcohol. The most permeable membranes were obtained by treatment with 97 per cent alcohol, but these membranes were extremely fragile and very apt to rupture.

All the membranes described by these various authors have a common disadvantage in that they become brittle and stiff when permitted to become completely dry and are then rendered impermeable and very easily broken. This necessitates the keeping of these membranes under water. The membranes described by the writer in this paper are extremely flexible even after being allowed to dry for a period of 2 weeks at room temperature and still retain their permeability. Some of these membranes have been rolled up in a ball like a wad of tissue paper without being injured in the slightest as is shown by the fact that when blown out and filled with a saturated solution of ammonium chloride they exhibited a high degree of endosmosis. A difference in level of 25 cm. in a tube 5 mm. in diameter has been observed when such a membrane has been placed in distilled water for 5 minutes.

This remarkable flexibility in the dried collodion membranes is obtained by adding ethyl acetate to solutions of collodion in mixtures of absolute alcohol and dry ether. The method of preparation is as follows.

5 gm. of "Anthony's Negative Cotton," which has been dried for 48 hours over concentrated sulfuric acid, are placed in a clean and dry Erlenmeyer flask and 25 cc. of absolute ethyl alcohol are added, and the flask is agitated so that all the cotton is thoroughly moistened. 75 cc. of ether, which has been distilled from sodium, are now added and the flask is shaken until the cotton has completely dissolved. 15 cc. of ethyl acetate are now added with shaking to secure complete mixing of the solvents. The solution is allowed to stand over night and then the clear supernatant liquid is decanted off into another flask.

After preparing numerous solutions of varying amounts of solvents, the proportions of solvents given above have been found to give the best results. It has been found that increasing the amount of ethyl acetate at first increases the flexibility of the resulting membrane rapidly, but that the effect diminishes as the concentration is increased so that the maximum flexibility is reached at a concentration of about 40 per cent. It has also been found that increasing the concentration of the ethyl acetate tends to diminish the permeability of the membrane to a slight extent. Acetone also seems to decrease the permeability of the membrane in proportion to its concentration.

The membranes are prepared inside of test-tubes or Kjeldahl flasks and may be made of any desired size, but the smaller ones are more easily obtained free from defects and of a more uniform thickness. The solution is poured into the flask, which must be perfectly clean and dry, and the excess of collodion is allowed to drain back into the container by holding the flask at an angle of about 60° and slowly rotating it until the collodion no longer drips freely and then the flask is clamped upside down in a stand and left until it is completely dry. Drying may be hastened by blowing a gentle blast of air into the flask, or even better by inserting a tube, connected with a slight suction, into the flask, taking care not to touch the side of the vessel. When the membrane is perfectly dry it is removed by peeling the top of the film from the neck of the flask and then pouring a gentle stream of water between the membrane and the side of the flask. This frees the membrane and it can then be withdrawn. This method differs from those previously described in that the membrane is allowed to become perfectly dry before being placed in contact with water, while in previous methods the membrane must be immersed in water before it dries or it becomes impermeable.

The chief difficulty encountered by the writer was the appearance of pinholes in the membranes especially in those made in the larger flasks. These pinholes can be avoided by taking care that the flasks in which the membranes are cast are scrupulously clean and dry, and that the collodion solutions are allowed to settle until all the particles of dust and undissolved matter have collected at the bottom of the container and are then carefully decanted into a dust-free vessel. The membranes can be made more uniform in thickness by following the technique of Farmer (6).

The membranes are tested for pinholes and other defects by filling them with a strong solution of Congo red and placing them in a beaker of distilled water. Any leakage is easily detected by the appearance of the dye in the beaker. The permeability of the membranes is established by the readiness with which they allow potassium ferrocyanide to dialyze out from a normal solution into distilled water. No attempt has been made to determine the permeability of the membranes except as to whether they permit Congo red to pass or not. It is interesting to note, however, in this regard that a solution of Bence-Jones protein after being dialyzed against distilled water until free from salt gave no test for the protein in the original solution.

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STUDIES ON EXPERIMENTAL RICKETS.

XII. IS THERE A SUBSTANCE OTHER THAN FAT-SOLUBLE A ASSOCIATED WITH CERTAIN FATS WHICH PLAYS AN IMPORTANT RÔLE IN BONE DEVELOPMENT?

By E. V. McCOLLUM and NINA SIMMONDS,

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Plate 1.

(Received for publication, November 1, 1921.)

Mellanby (1) was the first to associate a protective function of certain fats with bone development. He observed that certain diets caused changes in the epiphyses of the long bones in dogs which appeared, insofar as could be detected through radiography, to be rachitic in nature. The inclusion of butter fat or cod liver oil led to the development of bones in which radiographs showed no abnormalities. He was thus led to postulate the theory that the dietary essential, fat-soluble A, or some substance which has a similar distribution in nature exerted an antirachitic effect.

In an earlier paper, we presented some evidence that there is in cod. liver oil a substance capable of exerting a favorable influence on bone growth which is distinct from fat-soluble A (antixerophthalmic substance (2)). We have pursued our investigations with a view to determining whether it is the fat-soluble A or some other substance which protects the skeleton when the calcium content of the diet is unfavorable to the formation of normal bone. In the present paper we present a series of records of groups of rats which were fed throughout the period of growth and over a considerable part of adult life on diets which were closely

comparable except that on one hand certain groups received butter fat, and on the other certain groups received cod liver oil. The defect in these diets, aside from the lack of or subminimal provision of a hypothetical bone-nourishing substance, was limited to deficiency of calcium. The phosphorus content was in every case not far from the optimal.

Under such regulated conditions of nutrition where the lack of calcium is very pronounced and the amount of phosphorus is adequate, the provision of a liberal amount of some organic substance which is associated with certain fats exerts a most remarkable effect in improving the well being of the animals. The problem we discuss in this paper is whether this substance is furnished by both butter fat and cod liver oil, and whether but one or more than one substance is involved in the enhancement of diets low in fat-soluble A when either of these fats is added to such diets.

The line of evidence presented in the charts is as follows: On a diet such as we employed, young rats are much better nourished, when supplied with 1 per cent of cod liver oil than with 10 to 20 per cent of butter fat, as is shown by better growth, fertility, success in rearing young, and in length of life. This is true, notwithstanding, that 3 per cent of butter fat is ample for providing the animals with sufficient fat-soluble A and any other organic substance exerting a special effect on the bones, when the content of calcium in the diet is raised to approximately half the optimal. The provision of nearly seven times this amount does not exert much protection to the animals against the specific detrimental effect of lack of calcium when the content of the diet in this element is from one-fifteenth to one-fifth or sixth the optimal amount. 1 per cent of cod liver oil, on the other hand, seems to increase in a very remarkable manner the effectiveness with which the anatomic elements of the body tissues deal with a very low calcium supply. Our results indicate that there is no progressive benefit to the animals brought about by the exhibition of greater and greater amounts of butter fat. This would indicate that the effects are not proportional to the amount of this fat which is included in the diet. Furthermore, it appears that 1 to 2 per cent of cod liver oil supplies as much of the substance which exerts a peculiar influence on bone

growth as the animals can profit by even when the calcium supply is very low indeed. An extensive experience in feeding diets of the type here described has failed to reveal any evidence that a rat is benefited by the inclusion of more than 5 per cent of butter fat when the calcium and phosphorus are normal.

It is further shown from the experimental data recorded in the charts that as the calcium content of the diet is increased the differences between the effects of butter fat and cod liver oil tend to disappear, and vanish completely or nearly so when the content of calcium reaches as much as one-half or more of the optimal amount.

The results of this series of experiments were so consistent and decisive that we can deduce no other conclusion than that cod liver oil contains in abundance some substance which is present in butter fat in but very slight amounts, and which exerts a directive influence on the bone development and enables animals to develop with an inadequate supply of calcium much better than they could otherwise do. This substance is apparently distinct from fat-soluble A, which is essential for growth and which is associated definitely with the prevention of ophthalmia (keratomalacia).

We are continuing these investigations along other lines which are yielding results which bear directly on the confirmation or refutation of these conclusions.

Hart and Steenbock have recently published results which harmonize with the view we have expressed above. They found that the administration of butter fat did not influence favorably the calcium balance in a goat, whereas under the dietary conditions employed by them the feeding of cod liver oil promptly changed the calcium balance from negative to positive (3).

In presenting the experimental data contained in this paper we have expressed the belief that it tends strongly to support the view that there are two distinct organic factors operating in the nutrition of a mammal which are associated with certain fats. One serves especially to fortify the defensive mechanism of the body against the effects of lack of calcium. We are, however, already in possession of very definite data of entirely different kinds from those discussed above, which indicate clearly that similar effects can be induced in animals deprived of calcium by

illumination with sunlight as well as by the action of cod liver oil (4). This observation, together with an appreciation of the fact that much work of a very refined nature must still be carried out before we fully understand the problems of growth of the osseous tissues, leads us to point out at this time our belief that the formation of definite conclusions either from the work of others or from our own would best be deferred until other experimental work now developing is completed.

Chart 1. Lots 2925 and 2934 were fed on diets which were essentially alike except that one contained 10 per cent and the other 20 per cent of butter fat. Both diets contained not far from the optimal content of phosphorus, but contained only about one-fifteenth the optimal amount of calcium (about 0.400 gm. of phosphorus and 0.050 gm. of calcium). Other factors in the diets were satisfactorily constituted. The significant point which we wish to emphasize in connection with these records is that the animals grew very poorly and had very short lives, as contrasted with animals fed a similar mixture containing 0.5 per cent of calcium carbonate and but 3 per cent of butter fat (Chart 16). This shows that the limiting factor in the growth of these animals was the calcium supply. This demonstrates that all factors other than calcium are so constituted as to fully meet the nutritive needs of the growing rat when sufficient calcium is available. Although some animals in Lot 2925 lived some weeks longer than the average in Lot 2934, we regard this as of no significance in connection with differences in butter fat content. Animals on diets of this type are in a condition of nutritional instability and the length of life of any individual as compared with another will depend on its natural vitality. When compared with Lots 2732 and 2733 (Chart 2) these records show that 10 to 20 per cent of butter fat fails to protect the growing rat against the effects of lack of sufficient calcium in the same manner as does 1 to 3 per cent of cod liver oil.

Chart 2. Lots 2732 and 2733 were fed diets essentially comparable in their properties to those described in Chart 1, except that the former received 1 and 3 per cent respectively of cod liver oil instead of 10 to 20 per cent of butter fat. Both diets contained essentially the same content of phosphorus and of calcium (0.390 gm. of phosphorus and 0.050 gm. of calcium per 100 gm. of food), as did those of Lots 2925 and 2934.

It is a very remarkable thing that the animals in these two groups grew much better than those in Chart 1. They were fairly fertile. Lots 2925 and 2934 were sterile. Lots 2732 and 2733 were fairly successful in rearing their young to the weaning age, soon after which age the young died. These animals presented a fairly well nourished appearance, whereas Lots 2925 and 2934, which received high intakes of butter fat, were very poorly developed and were of abnormal form (stocky), dirty and rough coated.

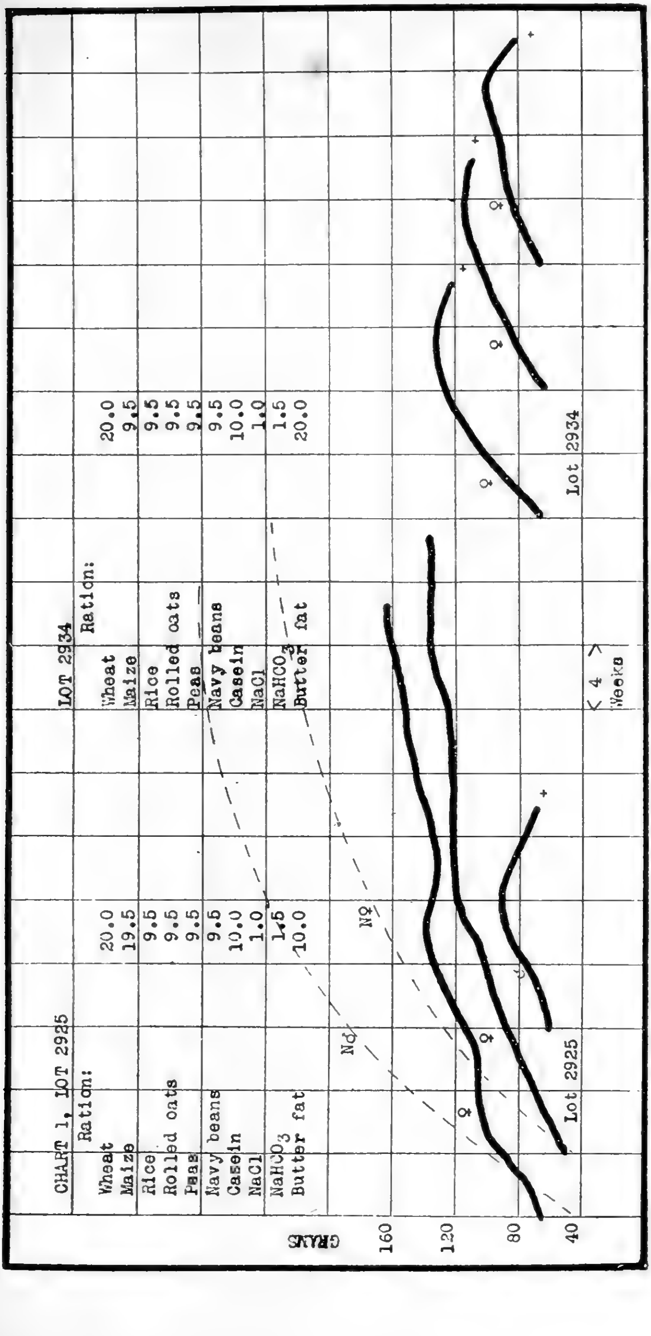
CHART 1. LOT 2925
Ration:
Wheat 20.0
Maize 19.5
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
NaCl 1.0
NaHCO3 1.5
Butter fat 10.0
LOT 2934
Ration:
Wheat 20.0
Maize 9.5
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
NaCl 1.0
NaHCO3 1.5
Butter fat 20.0
GRAMS
160
120
80
40
N♂
N♀
Lot 2925
Lot 2934
< 4 >
Weeks

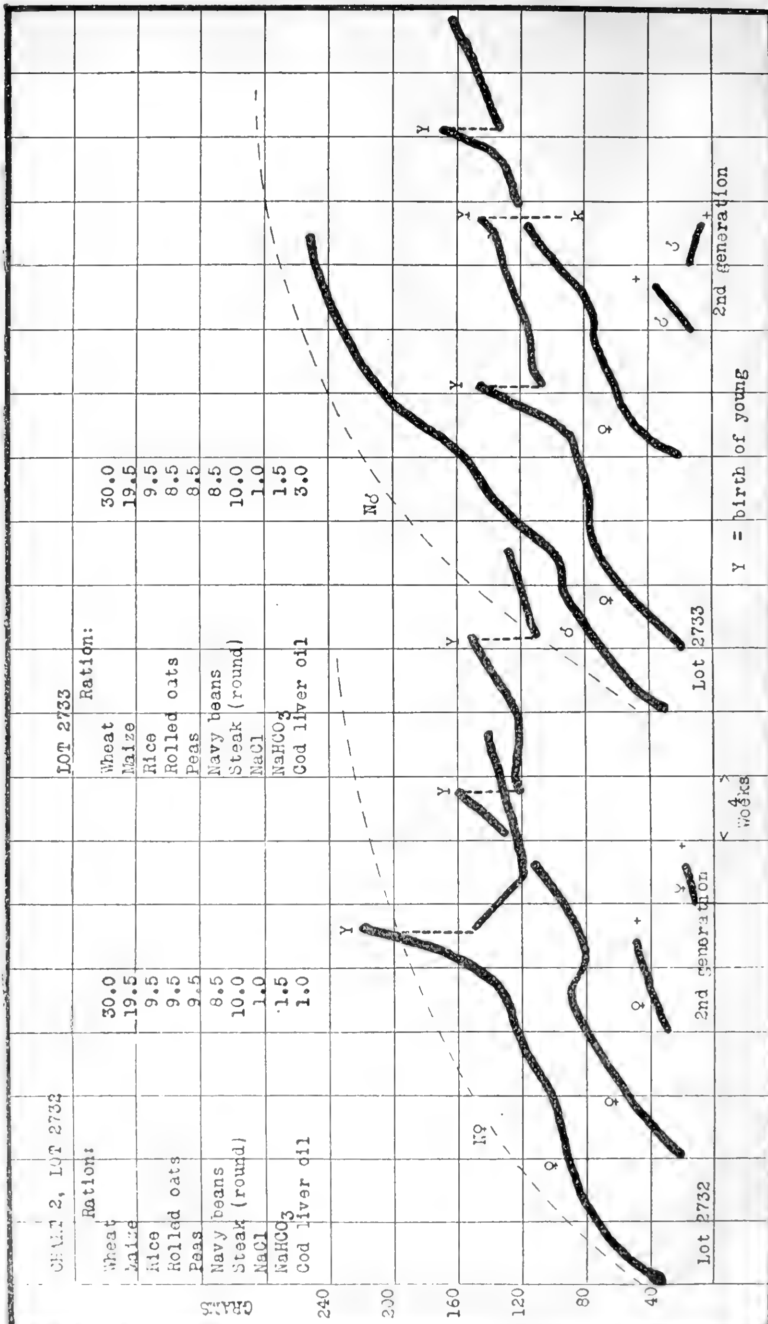
CHART 2, LOT 2732
Ration:
Wheat 30.0
Maize 19.5
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 8.5
Steak (round) 10.0
NaCl 1.0
NaHCO3 1.5
Cod liver oil 1.0
LOT 2733
Ration:
Wheat 30.0
Maize 19.5
Rice 9.5
Rolled oats 8.5
Peas 8.5
Navy beans 8.5
Steak (round) 10.0
NaCl 1.0
NaHCO3 1.5
Cod liver oil 3.0
GRAMS
240
200
160
120
80
40
N♀
N♂
Y
♀
♂
2nd generation
4 weeks
Lot 2732
Lot 2733
Y = birth of young

Cod liver oil, we have repeatedly observed with different diets, protects growing rats against the injurious effects of lack of calcium and enables them to grow and appear well nourished for a considerable period, where they would fail to grow and would be very inferior with even much greater amounts of butter fat instead of the cod liver oil.

It is especially remarkable that whereas 3 per cent of butter fat suffices when added to food mixtures of the type under discussion to meet all the requirements of the rat for fat-soluble A and for the specific calcium-depositing factor, if there be one, when the diet contains favorable concentrations of calcium, 10 to 20 per cent of butter fat fails to shield them in a manner at all comparable with 1 per cent of cod liver oil. Again, it is remarkable that animals specifically fasted for calcium are protected as effectively by 1 per cent of cod liver oil as by 3 per cent or higher planes of intake.

We do not believe that our samples of butter fat could differ in the concentration of the calcium-depositing substance, since, as will be shown in later charts, when the diet contains calcium in amounts ranging from 0.1 to 0.5 per cent of the carbonate, butter fat becomes adequate to supply the nutritive needs of the body and the differences in the effects of cod liver oil and of butter fat in the diet tend to disappear.

Chart 3. Lot 2821 was fed a diet essentially comparable to those discussed in the preceding charts except that its content of 20 per cent of casein raised the phosphorus content of the food mixture. Like the preceding diets it was very poor in calcium. 5 per cent of butter fat did not suffice to protect the animals against lack of calcium. This chart should be compared with Chart 4, Lot 2822, which differed significantly only in containing 2 per cent of cod liver oil and 3 per cent of butter fat.

Lot 2821 was very poorly nourished and became badly deformed. The coats of these rats were rough and thin (see Fig. 1) and they aged very early.

Chart 4. Lot 2822 had a diet exactly similar to that of Lot 2821 (Chart 3) except that 2 per cent of cod liver oil replaced 2 per cent of butter fat. This modification of the diet made a remarkable difference in their growth and well being (see Fig. 2). The former were stunted, infertile, and short lived. The latter grew to full normal size, presented a well nourished appearance, and were fairly fertile, and succeeded in rearing a considerable number of their young to the weaning age. The young were puny, pot-bellied, almost completely stunted in growth, and died early. The mothers declined rapidly after nursing two or three litters. The males, while well nourished for an interval following the completion of growth, soon presented a poorly nourished appearance and aged early.

The effect of the cod liver oil was to make the animals in some degree immune for a time to the injurious effects of lack of calcium. Even ten times as much butter fat could not do this. If a small addition of calcium were made to this diet the butter fat would supply sufficient of some organic

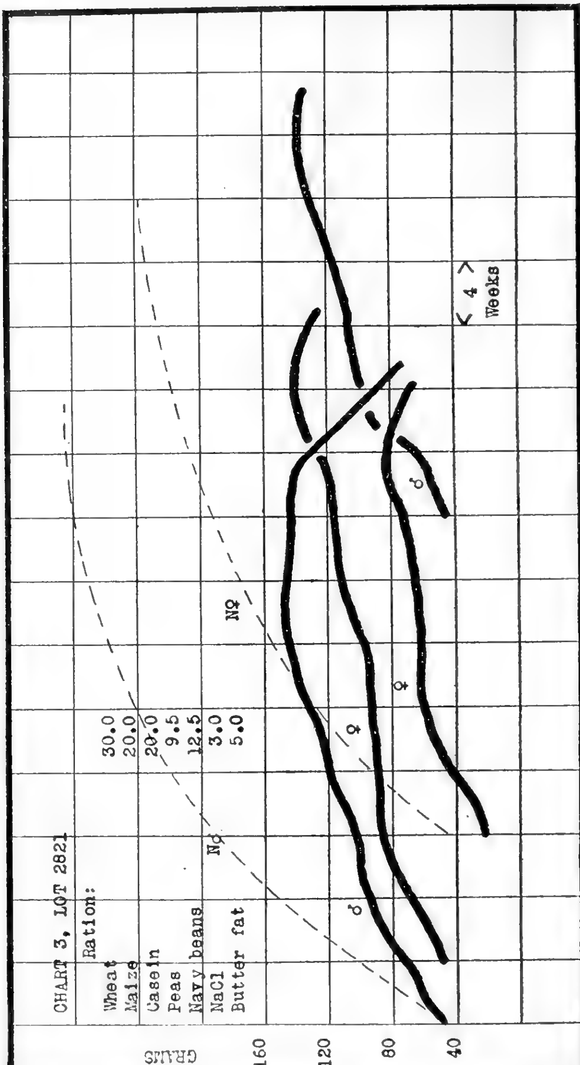
CHART 3, LOT 2821
Ration:
Wheat 30.0
Maize 20.0
Casein 20.0
Peas 9.5
Navy beans 12.5
NaCl 3.0
Butter fat 5.0
GRAMS
160
120
80
40
< 4 >
Weeks
N♂
N♀
♂
♀
♀
♂

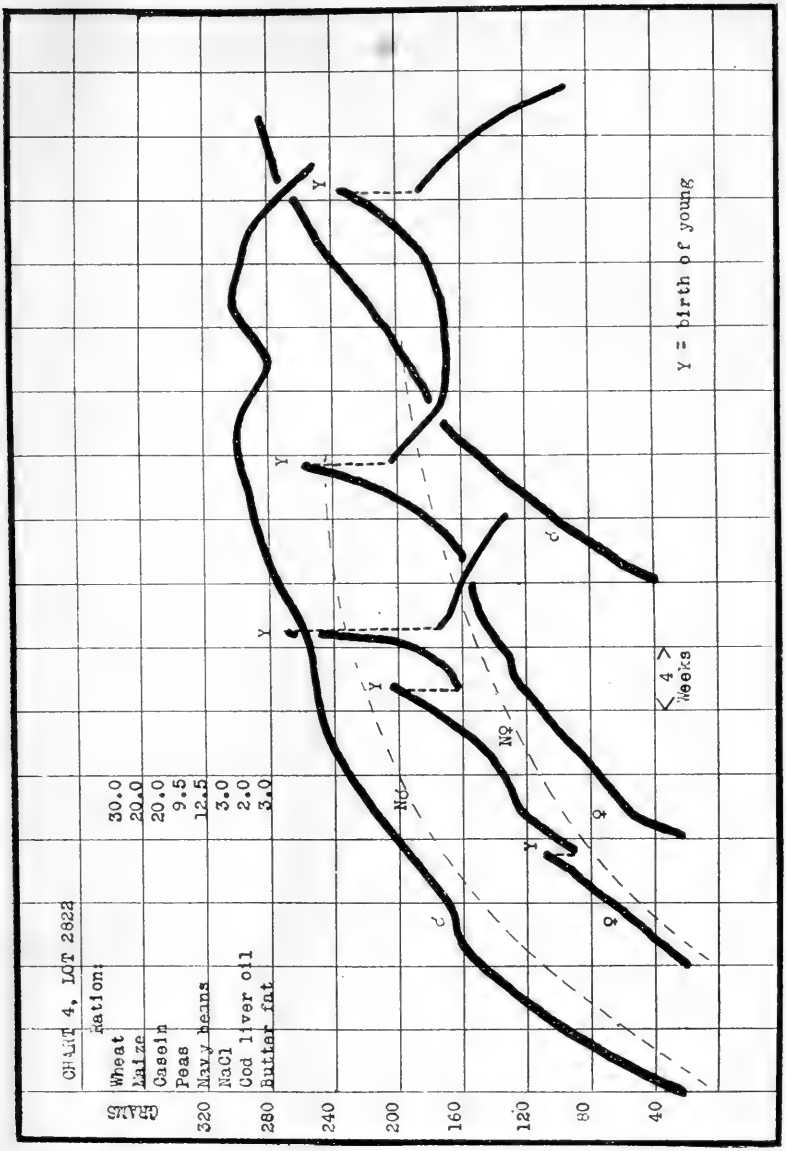
CHART 4, LOT 2822
Ration:
Wheat 30.0
Maize 20.0
Casein 20.0
Peas 9.5
Navy beans 12.5
NaCl 3.0
Cod liver oil 2.0
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
4 Weeks
N♂
N♀
♂
♀
Y

substance intimately associated with the development of the osseous system, and the animals would develop normally. In order to differentiate between the nutritive value of cod liver oil and of butter fat *in a qualitative way* it must be administered with a diet poor in calcium, for with an adequate supply of calcium and phosphorus in the food either serves equally well to supplement such diets as are here discussed.

Chart 5. Lot 2766. This and succeeding groups of experimental animals illustrate the comparative value of cod liver oil and of butter fat in the presence of suboptimal amounts of calcium, where the phosphorus content of the diet is near the optimum. A small amount of calcium (0.045 gm.) was added in the 5 per cent of milk powder but this was too small to contribute much to the well being of the animals.

These records should be compared with those of Chart 6 (Lot 2765), whose diet was almost identical except that it contained 1 per cent of cod liver oil instead of 10 per cent of butter fat. The rats were protected in a remarkable way against the effects of lack of calcium by this small amount of cod liver oil. 10 per cent of butter fat with this diet afforded some protection, but although this amount is at least three times that required to meet all the needs of the rat for protection against ophthalmia and to enable it to develop a normal skeleton when the diet contains the optimal amount of calcium, it fails to supply enough of some substance intimately concerned with bone formation when the calcium intake is low. These results point to the existence of a specific calcium-depositing substance distinct from fat-soluble A (antixerophthalmic substance).

On this diet the animals grew slowly but never attained the full adult size. The females were capable of producing several litters each but the infant mortality was high and they early developed signs of senility. The second generation confined to this food supply was greatly stunted and inferior. Their forms were very short and stocky. They had large deposits of body fat.

Chart 6. Lot 2765 should be contrasted with Lot 2766 (Chart 5). The significant difference in the composition of the diets of these two groups was in the nature of the fats which they contained. Both diets were far below the optimal in their content of calcium, but were otherwise well constituted. 1 per cent of cod liver oil very effectively protected these animals against the harmful effects of calcium starvation because of its content of some organic substance which appears to be distinct from that substance (fat-soluble A) which is essential for growth and is a specific agent in preventing ophthalmia of dietary origin. The protection afforded by the cod liver oil is not complete. It consists in enabling growth to proceed and causes the animals to appear externally to be well nourished. This is very apparent when we contrast the animals described in Chart 5, which had the same diet with 10 per cent of butter fat, with those in Chart 6, which had 1 per cent of cod liver oil. This contrast we have repeatedly observed in rats fed other diets low in calcium where fat-soluble A was in one case supplied by cod liver oil and in the other by butter fat.

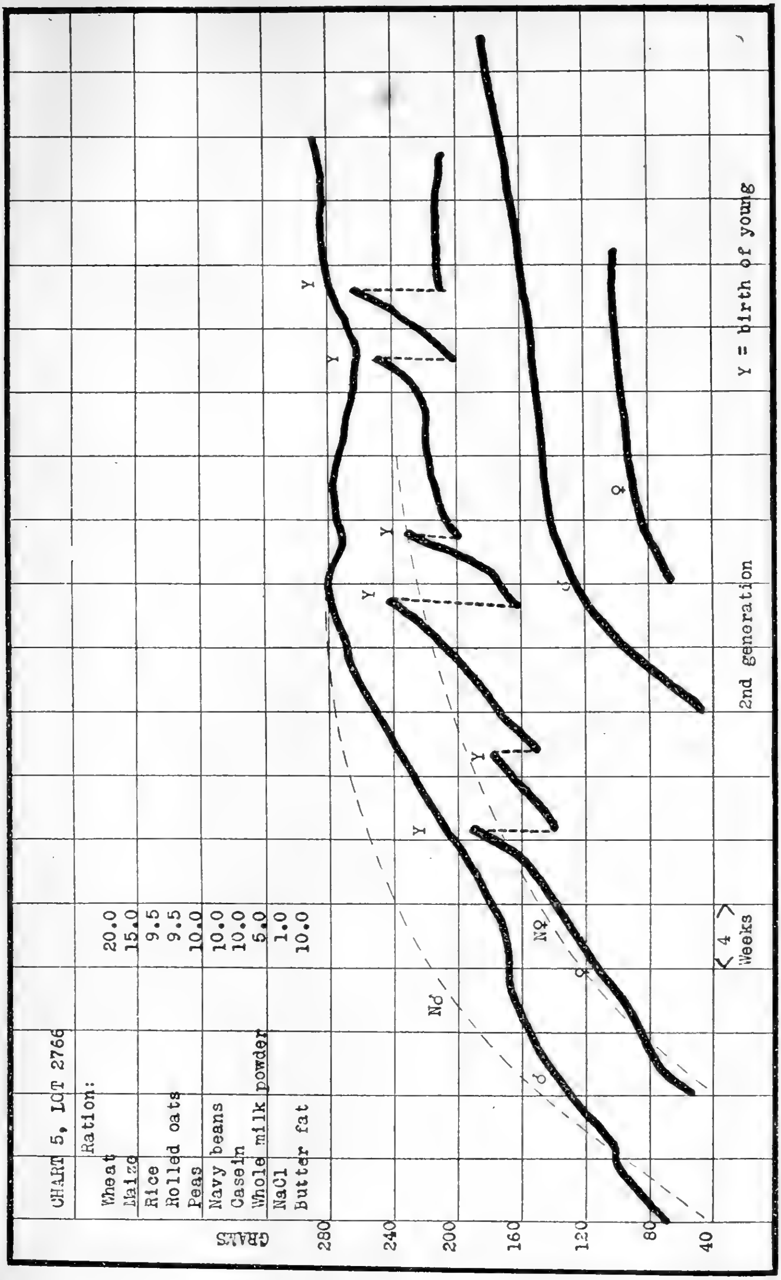
CHART 5, LOT 2766
Ration:
Wheat 20.0
Maize 15.0
Rice 9.5
Rolled oats 9.5
Peas 10.0
Navy beans 10.0
Casein 10.0
Whole milk powder 5.0
NaCl 1.0
Butter fat 10.0
GRAMS
280
240
200
160
120
80
40
Y = birth of young
2nd generation
4 Weeks
N♂
N♀
♂
♀
Y

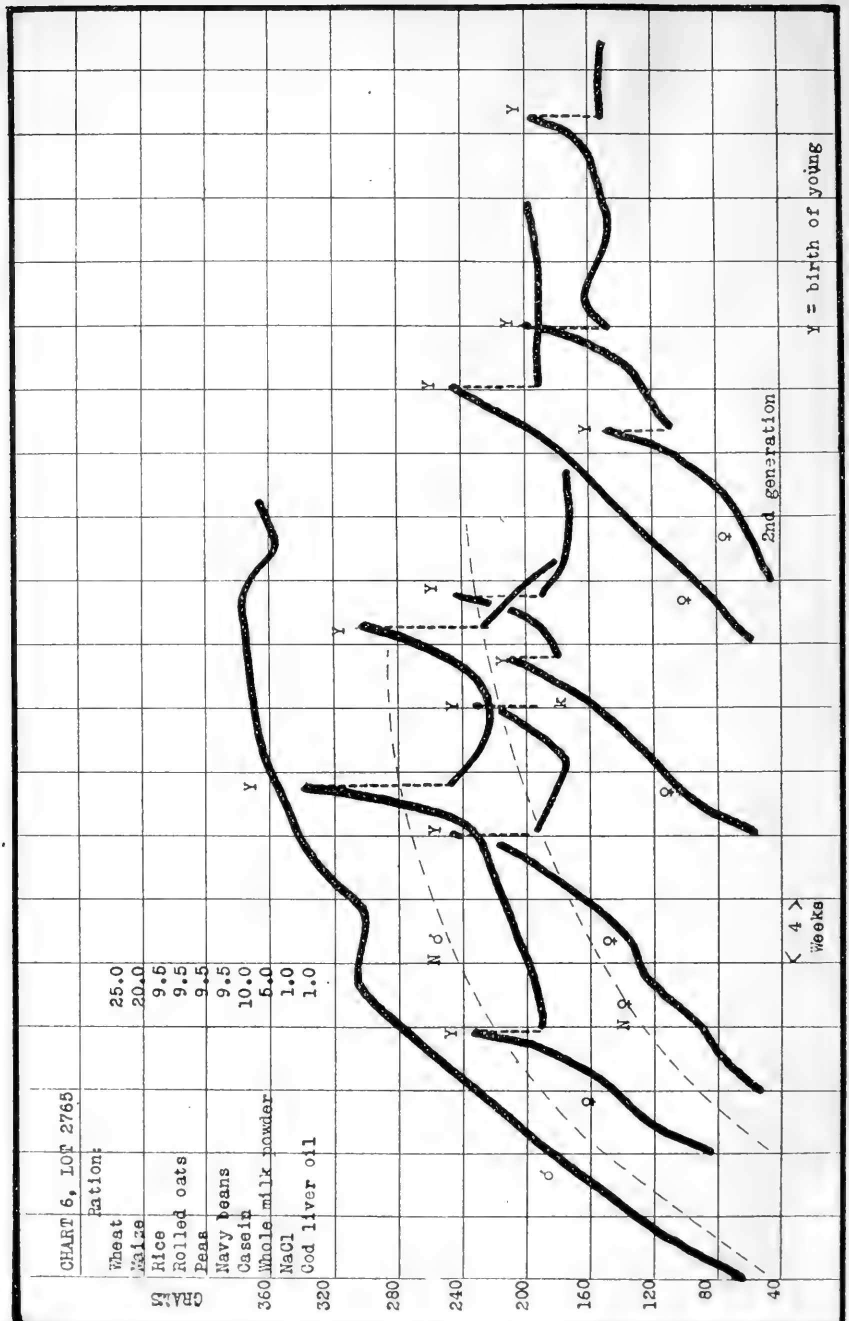

CHART 6, LOT 2765
Ration:
Wheat 25.0
Maize 20.0
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
Whole milk powder 5.0
NaCl 1.0
Cod liver oil 1.0
GRAMS
360
320
280
240
200
160
120
80
40
< 4 > Weeks
Y = birth of young
2nd generation
N ♂
N ♀
♂
♀
Y

That the animals receiving cod liver oil were in a state of nutritional instability notwithstanding their good external appearance and fertility, is shown by the tendency of the females to collapse and die while nursing a second or third litter of young. Their skeletons were very poorly calcified and when the animals were boiled in water it was impossible to separate even the bones of the pelvis. The femur remained intact but was frequently deformed in second or third generation animals. The skull bones disintegrated in this treatment.

Succeeding generations tend to fail on this diet, but not as rapidly as rats on similar diets containing butter fat even in large amounts (ten to twenty times as much fat as in the cod liver oil diets). The small amount of calcium added in the 5 per cent of milk powder exerted an observable effect in improving the well being of these rats. This is easily seen by comparing Lots 2732 and 2733 (Chart 2) with Lot 2765 (Chart 6).

Chart 7. Lots 2957 and 2958. These groups, and those described in later charts, illustrate the beneficial effects of adding small amounts of calcium to the standard diet employed in most of the experiments here described. A comparison of Chart 1 with Chart 7 shows how marked is the effect in promoting the vitality of rats by the addition of even 0.1 to 0.2 per cent of calcium carbonate to the diet. Lot 2957 failed to grow as well with 0.1 per cent as did Lot 2958 with 0.2 per cent addition. The contrast is much greater in the vigor and capacity to grow of the young produced by these two groups. 0.2 per cent of calcium carbonate made it possible for the second generation to develop fairly well and produce young, whereas the addition of but 0.1 per cent did not enable the young appreciably to grow or to extend their lives beyond about 60 days after weaning. Both groups aged early and had abnormal forms. They were short and stocky. Even 1 per cent of cod liver oil makes rats on this diet develop long, lithe forms, although they fail early.

Charts 8 and 9. Lots 2952 (Chart 8) and 2953 (Chart 9) should be compared with Chart 7. The diets were the same except that in Chart 7 the fat addition consisted of 8 per cent of butter fat, whereas in Charts 8 and 9 it was 2 per cent of cod liver oil. Lot 2952 had 0.1 and Lot 2953 had 0.2 per cent of calcium carbonate added. The great superiority of cod liver oil over five to ten times as much butter fat is easily seen.

The general appearance of these animals was superior to the animals fed butter fat. Fertility was high and the mortality was low, although the nursing period was frequently prolonged beyond the normal time. The young did not look sleek and well nourished while depending on the mother, but later when placed on the family diet greatly improved in appearance.

Charts 10 and 11. Lot 2959 (Chart 10) should be contrasted with Lot 2954 (Chart 11). These show the growth and fertility of rats fed the basal diet discussed in preceding charts to which 0.3 per cent of calcium carbonate was added. In the former, 8 per cent of butter fat was added, whereas 2 per cent of cod liver oil was added to the latter. Both groups grew well,

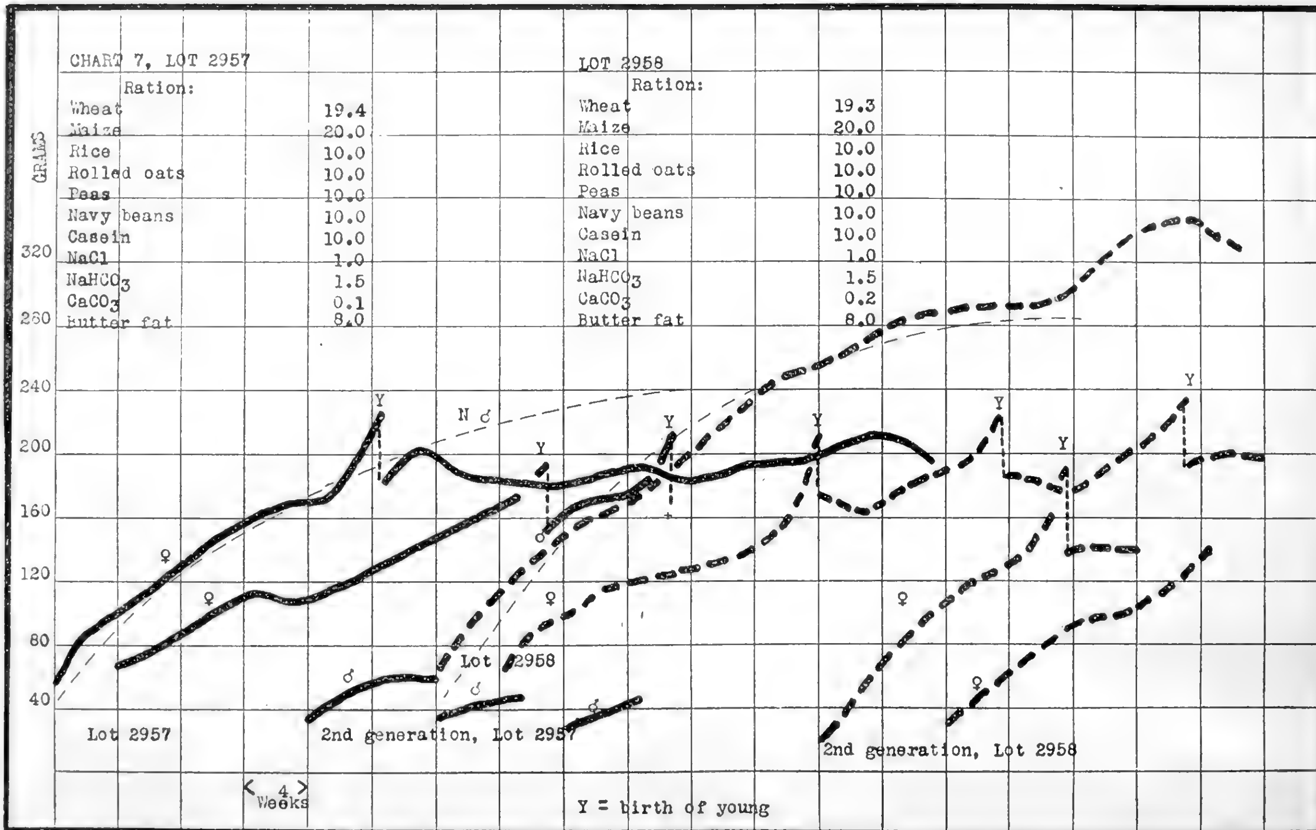
CHART 7, LOT 2957
Ration:
Wheat 19.4
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.1
Butter fat 8.0
LOT 2958
Ration:
Wheat 19.3
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.2
Butter fat 8.0
GRAMS
320
280
240
200
160
120
80
40
N ♂
Y
♀
♂
Lot 2958
Lot 2957
2nd generation, Lot 2957
2nd generation, Lot 2958
< 4 >
Weeks
Y = birth of young

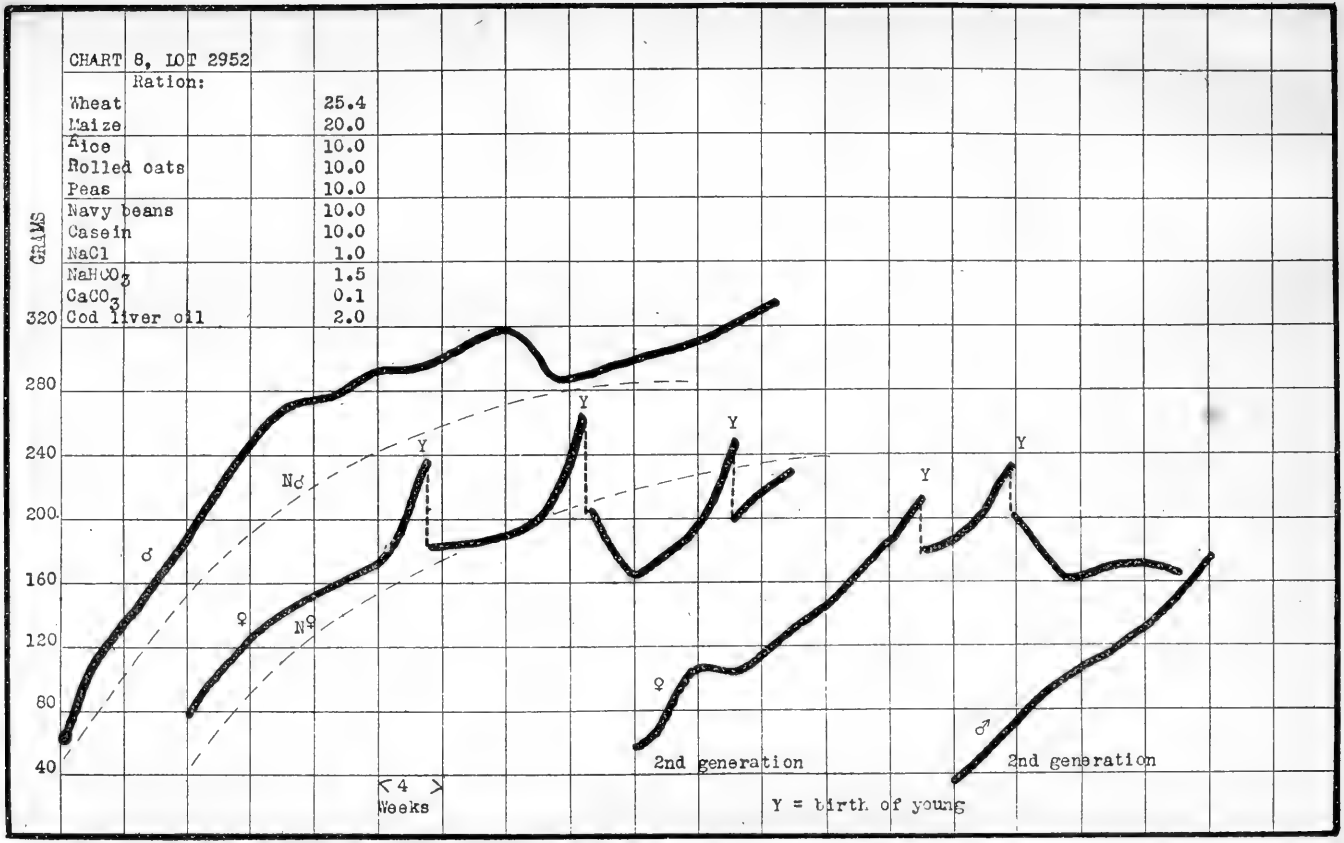
CHART 8, LOT 2952
Ration:
Wheat 25.4
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.1
Cod liver oil 2.0
GRAMS
320
280
240
200
160
120
80
40
♂
♀
N♂
N♀
Y
Y
Y
Y
Y
♀
♂
2nd generation
2nd generation
<4>
Weeks
Y = birth of young

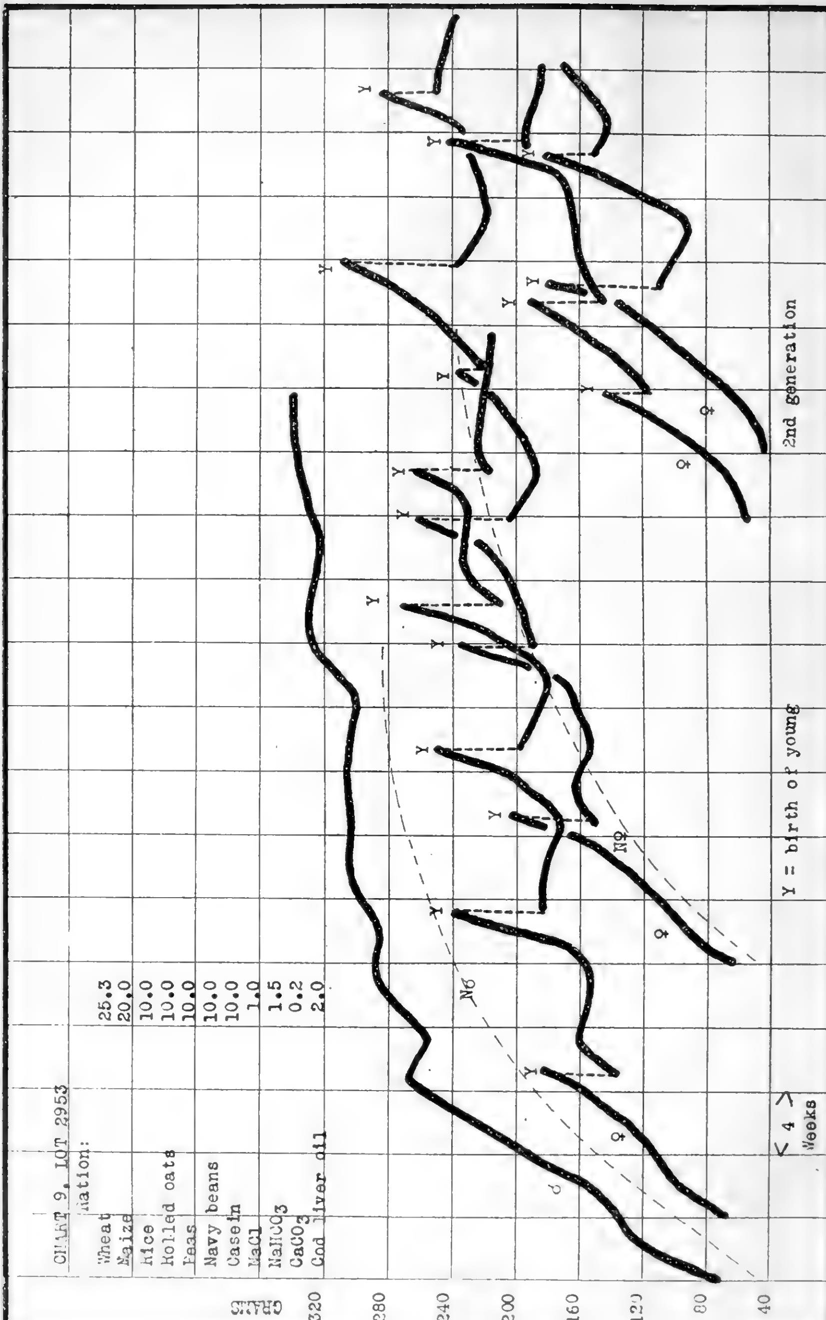
CHART 9. LOT 2953
Ration:
Wheat 25.3
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
$NaHCO_3$ 1.5
$CaCO_3$ 0.2
Cod liver oil 2.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
2nd generation
4 Weeks
♂
♀
N♂
N♀
Y

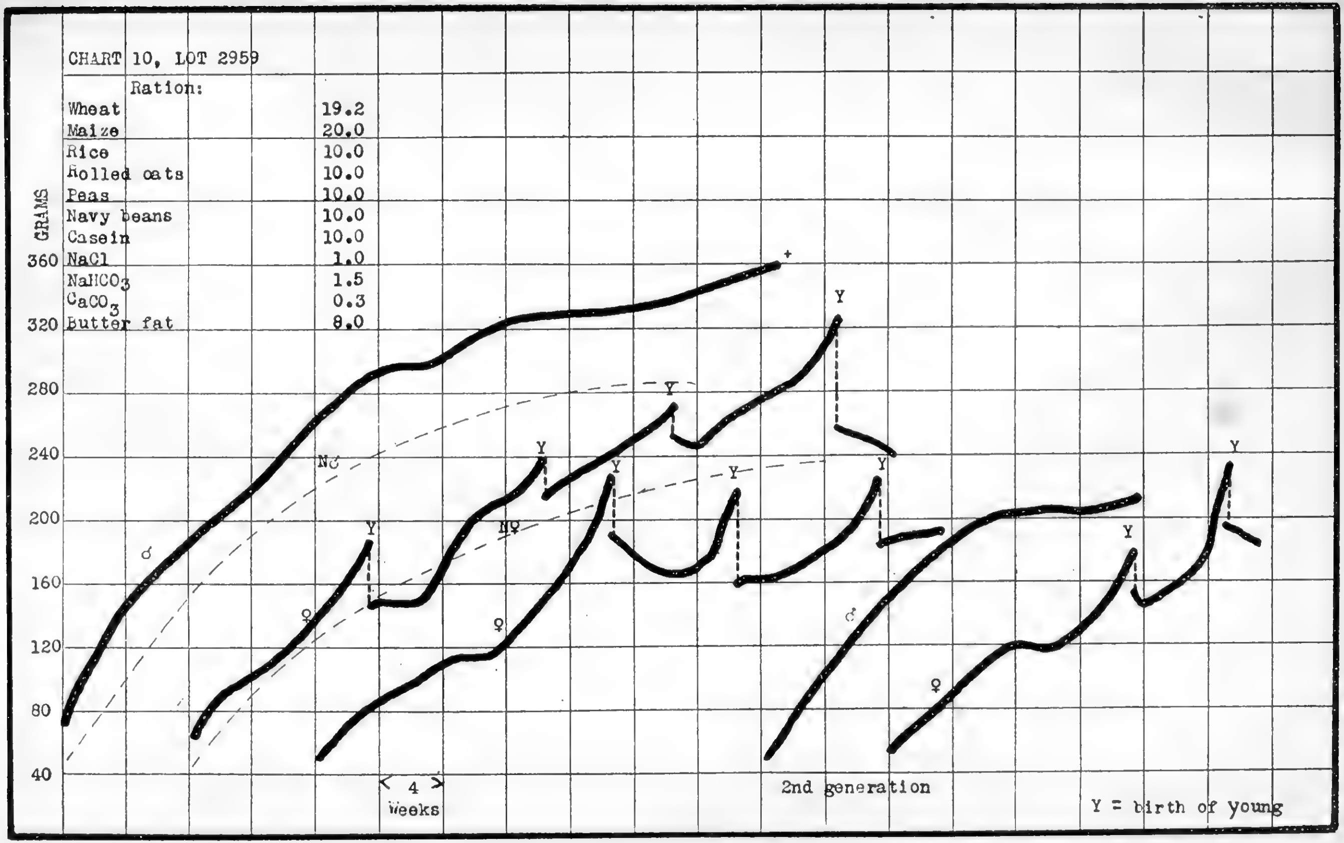
CHART 10, LOT 2959
Ration:
Wheat 19.2
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
NaHCO3 1.5
CaCO3 0.3
Butter fat 8.0
GRAMS
360
320
280
240
200
160
120
80
40
N♂
N♀
♂
♀
Y
< 4 >
Weeks
2nd generation
Y = birth of young

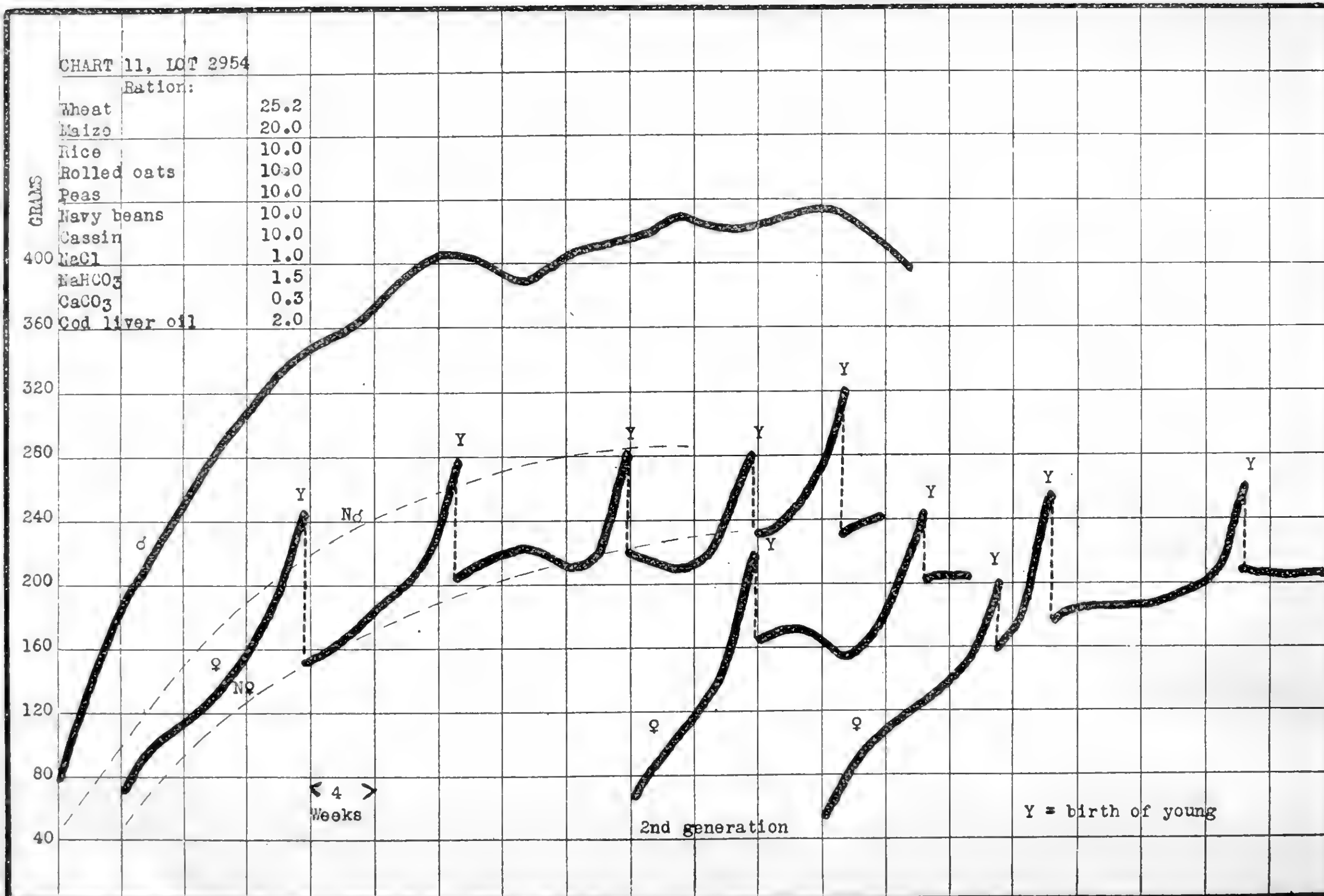
CHART 11, LOT 2954
Ration:
Wheat 25.2
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Cassin 10.0
NaCl 1.0
NaHCO3 1.5
CaCO3 0.3
Cod liver oil 2.0
GRAMS
400
360
320
280
240
200
160
120
80
40
♂
♀
N♂
N♀
Y
4
Weeks
2nd generation
Y = birth of young

but it is easy to see that the group receiving the cod liver oil was superior to those getting butter fat. It is apparent from these and preceding charts that as higher additions of calcium are made to this otherwise satisfactory diet, the difference between the dietary qualities of butter fat and cod liver oil tend to disappear. This is further emphasized in Charts 12 to 15, in which higher planes of calcium intake were furnished.

Fertility was high in both these groups and rearing of the young was the rule. In these respects the cod liver oil group was more successful than the butter fat group. There was a corresponding difference in the smartness of their appearance.

Charts 12 and 13. Lots 2960 and 2955 correspond in every way to the two groups described in Charts 10 and 11, except that 0.4 per cent of calcium carbonate was added in each case. One diet contained 8 per cent of butter fat and the other 2 per cent of cod liver oil. The well being of these two groups presented less contrast than did those in Charts 10 and 11, but it was still possible to detect that those receiving cod liver oil were better nourished than those getting butter fat. This was shown especially in the behavior of the second generation, those from the cod liver oil achieving greater size and presenting a smarter appearance than those from the butter fat group. The animals described in Charts 10 to 16 inclusive showed essentially the same degree of solicitude in caring for their immature young.

Charts 14 and 15. Lots 2961 and 2956 continue the series now under discussion. They received 0.5 per cent of calcium carbonate in each case, and Lot 2961 had 8 per cent of butter fat, whereas Lot 2956 had 2 per cent of cod liver oil. With this moderate addition of calcium, which represents diets containing less than half the optimal content of this element, the differences in the supplementary value of butter fat and cod liver oil practically disappear. Both groups appeared to be nearly equally capable of growth and fertility and succeeded about equally well in rearing their young. The succeeding generations, including the third, showed but slight differences in vitality. There was, however, a slight advantage in favor of the cod liver oil.

Chart 16. Lot 2839 completes the argument we are presenting, to the effect that there are differences in the dietary properties of cod liver oil and of butter fat, which we can explain only on the assumption that we are dealing with two uncharacterized dietary factors in butter fat and cod liver oil. One of these is the antixerophthalmic substance, fat-soluble A. The other we suggest is a substance which plays a more important rôle in influencing the anatomic elements in the osseous system and may be designated as a calcium-depositing or phosphorus-mobilizing factor. Butter fat is richer in fat-soluble A than in the calcium-depositing factor. Cod liver oil is exceptionally rich in both substances.

It has only been found possible up to the present time to demonstrate the differences between these fats by using diets poor in calcium, for with

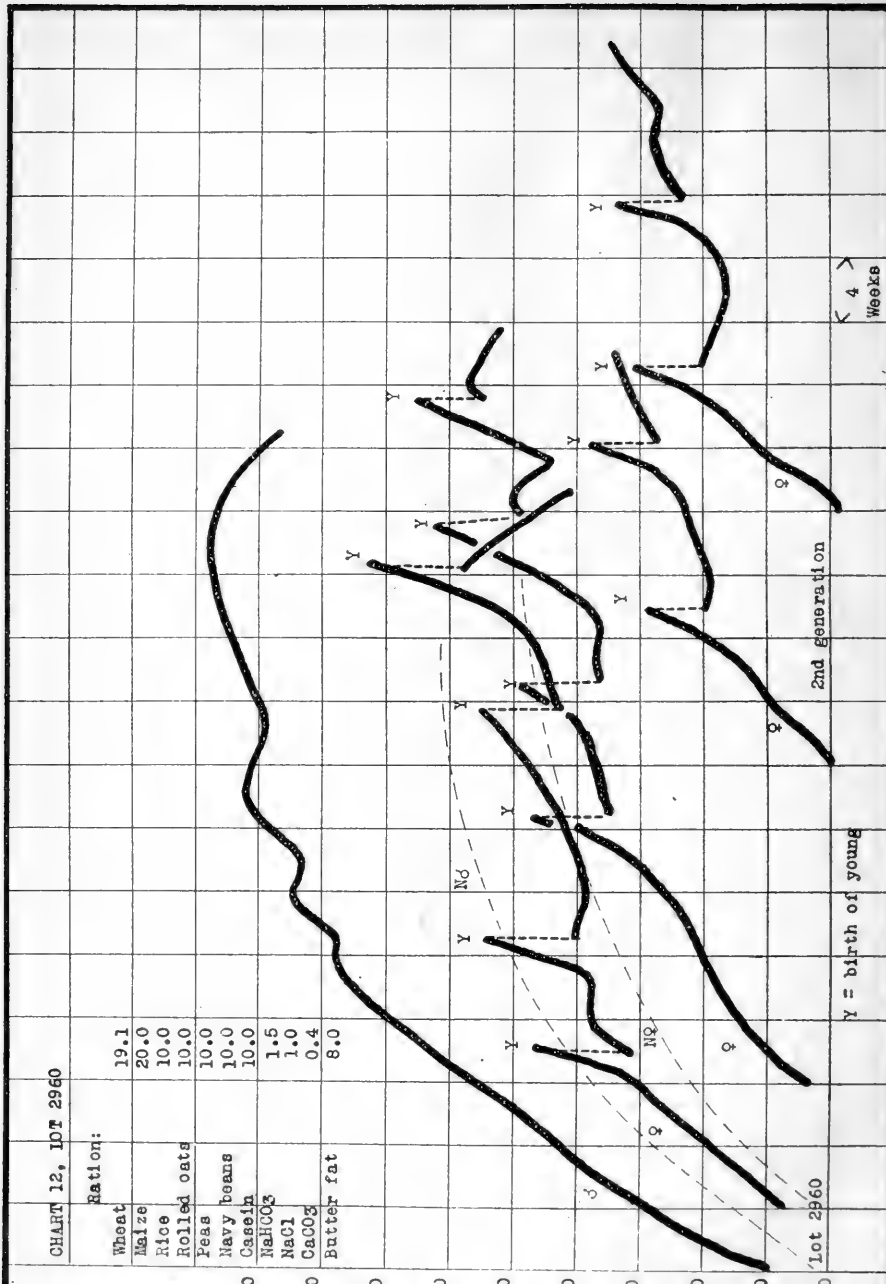

CHART 12, LOT 2960
Ration:
Wheat 19.1
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaHCO3 1.5
NaCl 1.0
CaCO3 0.4
Butter fat 8.0
GRAMS
400
360
320
280
240
200
160
120
80
40
Lot 2960
Y = birth of young
2nd generation
4 Weeks
N♂
N♀
♂
♀
Y

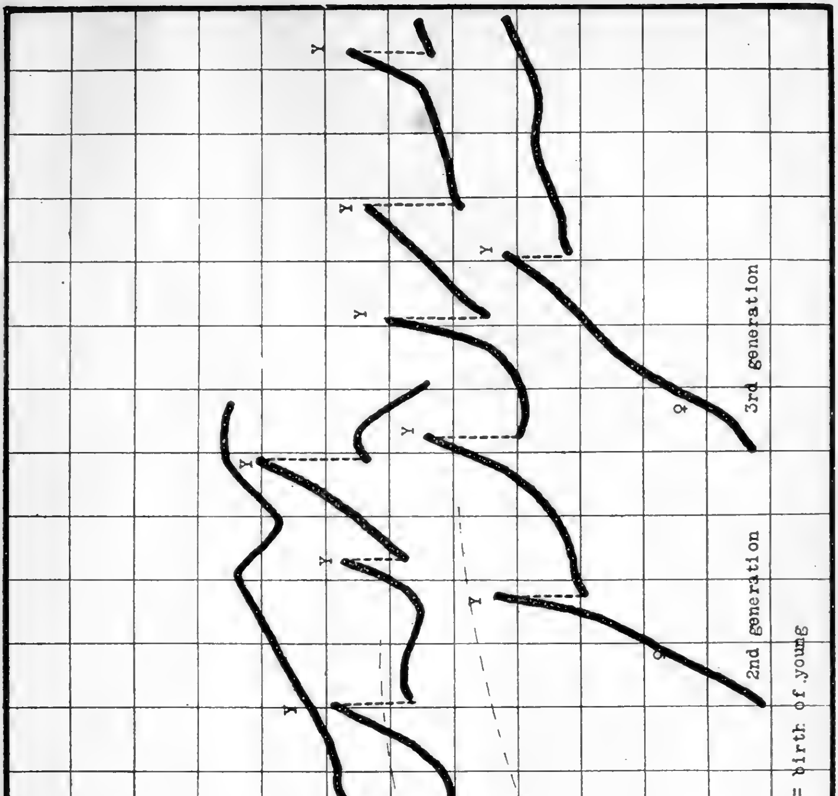
CHART 13. LOT 2955
Ration:
Wheat 25.1
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
NaHCO3 1.5
CaCO3 0.4
Cod liver oil 2.0
GRAMS
360
320
280
240
200
160
120
80
40
N♂
N♀
♂
♀
Y
2nd generation
3rd generation
Y = birth of young
4 Weeks

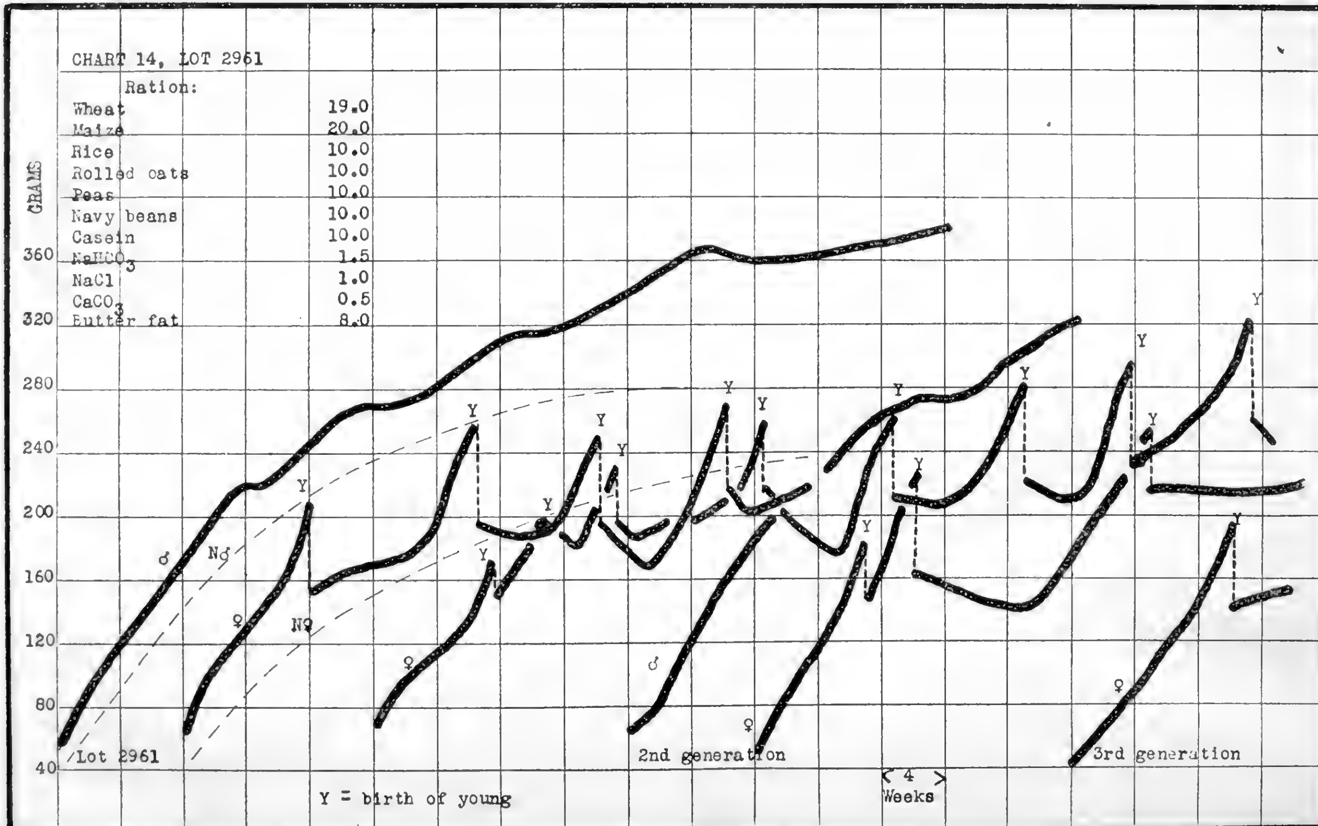
CHART 14, LOT 2961
Ration:
Wheat 19.0
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
$NaHCO_3$ 1.5
NaCl 1.0
$CaCO_3$ 0.5
Butter fat 8.0
GRAMS
360
320
280
240
200
160
120
80
40
Lot 2961
2nd generation
3rd generation
Y = birth of young
4 Weeks
N♂
N♀

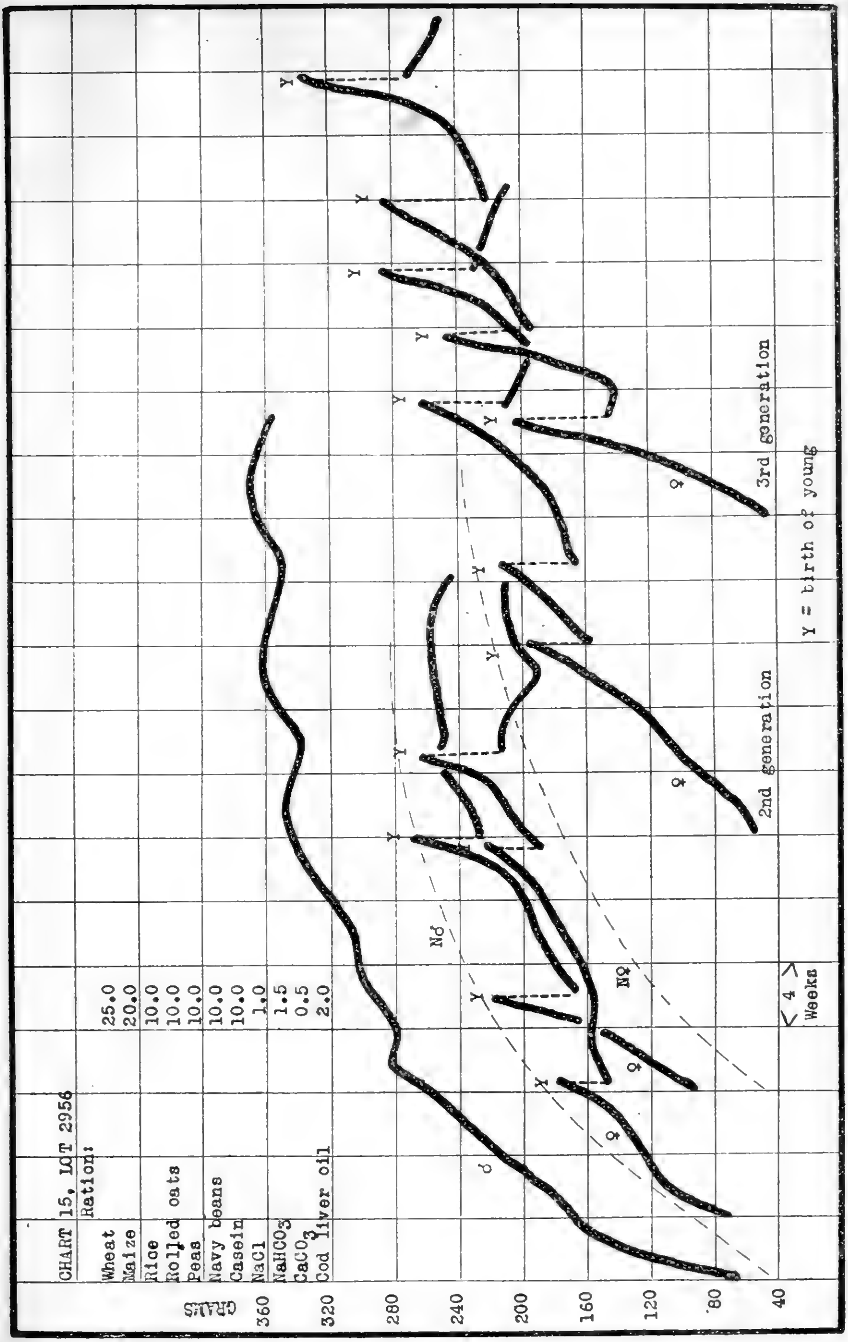

CHART 15, LOT 2956
Ration:
Wheat 25.0
Maize 20.0
Rice 10.0
Rolled oats 10.0
Peas 10.0
Navy beans 10.0
Casein 10.0
NaCl 1.0
NaHCO3 1.5
CaCO3 0.5
Cod liver oil 2.0
GRAMS
360
320
280
240
200
160
120
80
40
4 Weeks
N♂
N♀
2nd generation
3rd generation
Y = birth of young

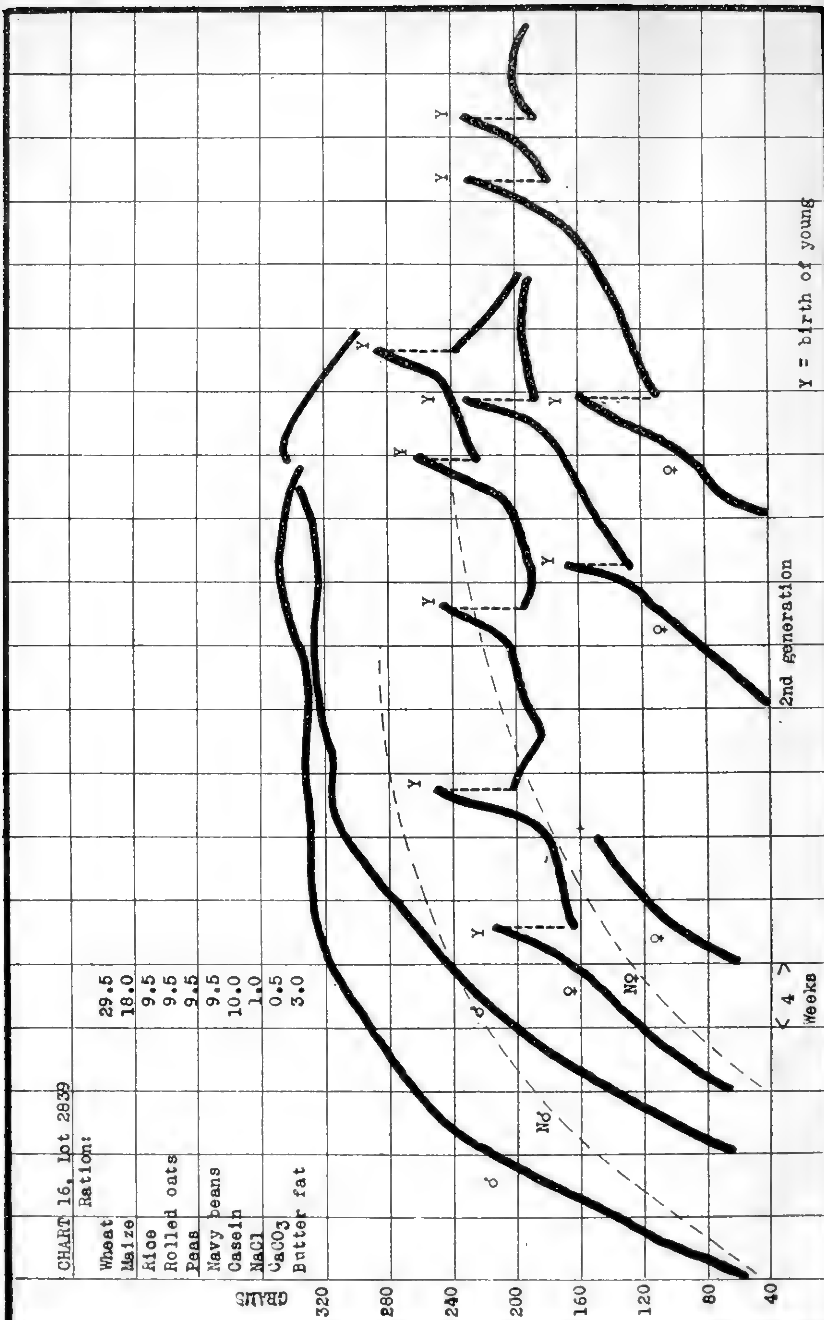
CHART 16, Lot 2839
Ration:
Wheat 29.5
Maize 18.0
Rice 9.5
Rolled oats 9.5
Peas 9.5
Navy beans 9.5
Casein 10.0
NaCl 1.0
$CaCO_3$ 0.5
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
2nd generation
< 4 > Weeks
Y
♂
♀
N♂
N♀

low intake of this element there is a correspondingly high requirement of the substance which we have spoken of as the calcium-depositing factor, but which is more than this. It exerts a distinct influence on the anatomic elements of the growing bone and enables the osteoblasts to form approximately the optimal amount of osteoid tissue. It leads to the deposition of calcium phosphate in a degree which is not possible in its absence when the calcium content of the diet is very low. It improves the general well being of the animals.

The most important point brought out by the records in this chart is that when 0.5 per cent of calcium carbonate was added to the diet, which still contained only about half the optimal content of calcium, even so small an amount of butter fat as 3 per cent sufficed to enable the animals to grow in a normal manner and to exhibit normal vitality as shown in fertility and success in rearing young. The second generation was somewhat undersized but was fertile.

This chart should be compared with Chart 1. Lot 2934 had nearly seven times the content of butter fat which was consumed by Lot 2839, yet in the absence of a calcium supply exceeding that furnished by a cereal and legume seed mixture they were not protected by this high butter fat intake.

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EXPLANATION OF PLATE 1.

Fig. 1 shows the appearance of a rat which was restricted to Diet 2821 (Chart 3). This diet was faulty in that its calcium content was too low and the phosphorus near the optimal. The fat in the diet consisted of 5 per cent of butter fat. Note the poor coat and general appearance of inferior development. This picture should be contrasted with Fig. 2, which had the same diet but with 2 per cent of cod liver oil and 3 per cent of butter fat.

Fig. 2. This rat from Lot 2822 was the same age as that shown in Fig. 1, and was confined to its experimenal diet for the same number of days (155). Its diet differed from that shown in Fig. 1 only in that 2 per cent of cod liver oil replaced 2 per cent of butter fat. Note the superior development, the good coat, and general appearance of physical well being brought about by the small amount of cod liver oil.

FIG. 1.

FIG. 2.

THE BIOGENESIS OF OIL OF PEPPERMINT.

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(*From the Wisconsin Pharmaceutical Experiment Station, Madison.*)

(Received for publication, October 25, 1921.)

It is commonly stated in the literature that the Japanese peppermint oil contains such a large proportion of menthol that it is a magma of crystals and saturated mother liquor even at ordinary temperature; that the European oils, on the other hand, have a minimum of free menthol; while the American oil is intermediate. The high menthol content of the Japanese oils has led to the introduction into Europe and America of strains of Japanese plants. The expectation that these would retain their chemical characteristics seems to have been justified in at least one instance.[1]

In studying the cohobated oils of American and Japanese peppermints grown by the Wisconsin Pharmaceutical Experiment Station, it was logically but a step further to expect that the Japanese mint oil would be exceedingly rich in menthol, and that the American mint oil would be composed of both menthol and menthone. This expectation was based on the fact that the oxygenated constituents of an oil are usually more soluble in water than the hydrocarbons, and hence are recovered from the aqueous distillate in relatively greater quantity by cohobation. Therefore, it was a distinct surprise to find that the 1920 cohobated oil of Japanese peppermint consisted almost wholly of pulegone;[2] and that the American mint oil, though having menthone[3] and menthol[4] as major constituents, contained methyl-1-cyclohexanone-3 as well.[5] The cohobated aqueous distillate

[1] See the Semiannual Report of Schimmel and Company, April, 1911, 92.

[2] Pulegone: Semicarbazone, M. P. 168° (recorded 167.5–168°).
Nitrosite, M. P. 83° (recorded 81.5°).

[3] Menthone: Semicarbazone, M. P. 185° (recorded 184°).

[4] Menthol: Pure crystals, M. P. 42.5°.

[5] Methyl-1-cyclohexanone-3: Semicarbazone, M. P. 180° (recorded 180°). The above experimental data were embodied in a paper presented to the New Orleans Meeting of the American Pharmaceutical Association, September, 1921.

was found to contain acetone.[6] The last two compounds are the products of hydrolysis of pulegone. Accordingly it was thought that both mints produced pulegone, but that it was subsequently hydrolyzed in the American mint by metabolic processes.

Further reflection on the problem and discussion with others emphasized the fact that in recent years the peppermints have been considered as hybrids rather than as true species or varieties. Whereupon the opportunity for the comparison of morphological with chemical hereditary characteristics seemed too good to pass by. At present, the American mint is considered to be a cross between *Mentha aquatica* Linné and *Mentha spicata* Hudson. The known chemical constituents of each oil and a possible scheme of their biogenesis is given below. The data are taken from Gildemeister.[7]

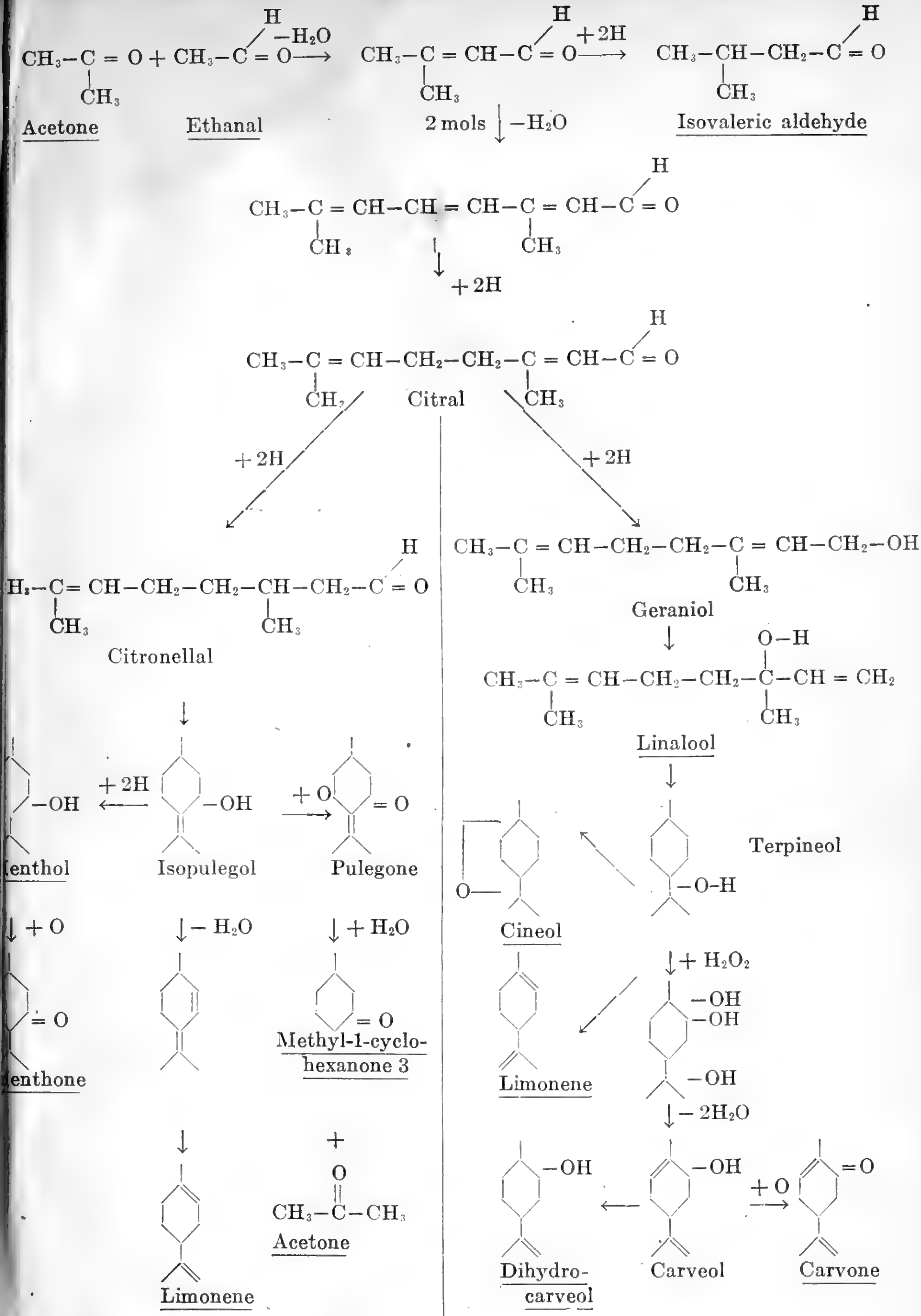
Mentha aquatica, Linné (*Mentha citrata,* Ehrhardt)		*Mentha spicata,* Hudson (*Mentha viridis,* Linné)

"American mint."

Mentha piperita, var. officinalis, forma rubescens, Camus.

A European oil reported to have a pulegone odor; one from Florida, that of linalool.	Acetaldehyde. Acetone. Methyl alcohol. Furfural. Isovaleric aldehyde. Amyl alcohol. Acetic acid. Isovaleric acid. Pinene. Phellandrene. Cineol. 1-Limonene. Menthol. Menthone. Methyl-1-cyclohexanone-3. Menthyl esters. Cadinene. A lactone. Dimethyl sulfide.	Acetic acid. Butyric acid. 1-Limonene. Phellandrene. Carvone. Dihydrocarveol (ester). Cineol.

[6] *Univ. Wisconsin Pharm. Exp. Station, Circular 2,* October, 1920, 17.
[7] Gildemeister, E., Die Aetherischen Oele, Leipsic, 2nd edition, 1910, iii.



Peppermint (*Mentha piperita*) *Spearmint* (*Mentha spicata*)

Compounds known to occur in the oils are underlined

This scheme or picture of how the oils of spearmint and peppermint may be elaborated offers, in spite of its tentative nature, numerous suggestions for speculation and experiment. Thus the striking parallelism in the relations of analogous compounds, for example of menthol and pulegone to isopulegol in the one mint and of dihydrocarveol and carvone to carveol in the other unmistakably suggests a common mechanism of formation. But most interesting is the thought that the Mendelian "factor," if such it is, which governs the formation of the carvone group in spearmint and of the menthone group in peppermint, lies in the conditions affecting the reduction of citral. For, as outlined, the two groups are separated at that point by a common type of reaction. And it is easy to conceive that the conditions which caused the reduction of the aldehyde group in spearmint were modified in cross-breeding to bring about the reduction of a carbon to carbon double bond in peppermint.

CONCLUSIONS.

Although it was found that the oils of American and Japanese peppermints are not strictly comparable in a botanical sense, still some interesting results are evident as a result of studying the problems originally suggested by that idea.

The constituents of the volatile oils of *Mentha piperita, var. officinalis* and *Mentha spicata* Hudson have been listed and compared.

A possible scheme of the biogenesis of the most important constituents has been drawn up.

It is evident that a great similarity exists in the reactions by which the menthol group on the one hand and the carvone group on the other are elaborated.

The two groups may have a common precursor, and are each derived from it by a reaction involving the addition of two atoms of hydrogen—the difference being only in the point of reduction.

The urgent need of a reliable examination of the volatile oil of *Mentha aquatica* becomes evident.

It should finally be emphasized that these data are presented only as a preliminary survey of what is known and as a glimpse of what may be. Also it is desired to emphasize the opportunity existing in the study of these common plants for the elucidation of phenomena which may be hereditary in character. The work is being continued.

THE CHEMISTRY OF THE OXIDATION OF SULFUR BY MICROORGANISMS TO SULFURIC ACID AND TRANSFORMATION OF INSOLUBLE PHOSPHATES INTO SOLUBLE FORMS.*

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Oxidation of Sulfur by Microorganisms.

When sulfur is added to unsterile soil, it is slowly oxidized to sulfuric acid; when the soil is previously sterilized, oxidation of the sulfur takes place only to a very limited extent depending upon the other chemical substances present. But when a sulfur-oxidizing organism is introduced, the sulfur is rapidly oxidized to sulfuric acid. This acid acts upon insoluble soil constituents such as calcium and magnesium carbonates, calcium silicates, and tricalcium phosphate, and brings them into solution. This process has been utilized by Lipman and associates (1916) for the transformation of the insoluble tricalcium phosphate into soluble forms by composting rock phosphate, sulfur, and soil to which the sulfur-oxidizing bacteria have been added. A few principles involved in these transformations, both by crude and pure cultures of the sulfur-oxidizing organisms, are set forth in this paper.

As a result of a series of studies, several organisms have been isolated, which are able to oxidize sulfur under various conditions. The oxidation of sulfur under acid and alkaline conditions seems to be affected by different groups of microorganisms. A detailed study of occurrence, morphology, and physiology of

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these organisms is found elsewhere (Waksman and Joffe, 1920–22). One of the most interesting organisms isolated by the authors is an aerobic, autotrophic, minute bacterium, *Thiobacillus thiooxidans* Waksman and Joffe, which is able to oxidize sulfur to such an extent as to reduce the hydrogen ion concentration of the medium to a pH of less than 1.0, even in the presence of buffering materials. It derives its energy from the oxidation of the sulfur and the carbon from the CO_2 of the atmosphere. The nitrogen can be supplied in the form of inorganic or organic materials.

In taking up the chemistry of the sulfur oxidation, attention must be called to the aerobic nature of the phenomenon.

$$\underset{64}{2S} + 2H_2O + \underset{96}{3O_2} = \underset{196}{2H_2SO_4}$$

Thus, for 64 units of sulfur, 96 units of oxygen are required to produce 196 units of sulfuric acid. The effect of oxygen is, therefore, of prime importance in the oxidation of sulfur.

Experiments with crude cultures of the organism reported elsewhere (Joffe, 1922) substantiate the theoretical suppositions based on the empirical equations involved in the chemistry of sulfuric acid. Mixtures of sulfur, rock phosphate, and soil inoculated with crude cultures of sulfur-oxidizing organisms were prepared; one set was aerated and the other left unaerated. The amount of phosphates brought into solution and the change in the hydrogen ion concentration, as expressed by the exponent pH of Sörensen, were used as criteria. The aerated mixtures were leading and, after 100 days, the percentage increase of soluble phosphates in the aerated over the non-aerated was 6 per cent, with a similar correlation in the increase of the hydrogen ion concentration. It is interesting to record here the fact that this biological process follows the laws of inorganic reactions. According to the mass law, the velocity of any reaction depends on the mass of the active ingredients involved and is at any moment proportional to the molecular concentration of the reacting components and a constant, which is characteristic of the chemical nature of the reacting substances. Whatever transformations the oxygen undergoes in the metabolism of the organism, the end-product is sulfuric acid; an increase in oxygen tension increases

the mechanism of oxidation of sulfur by the organisms. It is also possible that the oxygen from the air is not the only source; as pointed out above this particular organism derives its energy not from carbohydrates but from the oxidation of sulfur and is autotrophic in nature. Like green plants, the autotrophic organisms use the carbon dioxide from the air for structural purposes, but, unlike plants, these organisms accomplish it without the intervention of the photochemical reactions. The process of assimilation of carbon dioxide is accompanied by the splitting off of oxygen, which may also be used by the sulfur organisms in the process of oxidation.

Oxidation of Sulfur in the Ordinary Cultivated Soil.

Several typical experiments will be reported here to illustrate the mechanism of sulfur oxidation in the soil, both in the absence and in the presence of small and large amounts of rock phosphate. The sulfur and phosphate were added to the soil and well mixed. A crude well developed culture was used for inoculation. The moisture content of the soil was kept at an optimum by the addition of water at weekly intervals. The cultures were incubated at 25–27°C. The pH values were determined colorimetrically, according to the method of Clark and Lubs (1917); the phosphates and sulfates according to the method of the Official Agricultural Chemists (1916).

The results tabulated in Table I represent the oxidation of small amounts of sulfur in the soil. In this case 22.5 mg. of sulfur and 90 mg. of rock phosphate were added to 600 gm. of soil. The results tabulated in Table II represent the oxidation of large amounts of sulfur when introduced with large amounts of rock phosphate into the soil. In this case 30 gm. of sulfur and 90 gm. of rock phosphate were mixed with 480 gm. of soil in small pots.

When the course of change in reaction due to the sulfur oxidation, in the presence of tricalcium phosphate is studied, we find that the curve is regular till the pH reaches 2.8, then it becomes flat. This is a crucial point and, as long as there will be any phosphate left undissolved, the reaction will not go down very much, since at that point all the acid formed from the oxidation of the sulfur is used not in increasing the reaction of the medium, but in transforming the rock phosphate into soluble form. Once

the phosphates have been made soluble, the acidity begins to increase. This will be made clearer in the discussion of the sulfur oxidation by pure cultures in solution.

TABLE I.

The Oxidation of Small Amounts of Sulfur in the Soil.

Period of incubation.	pH value.
days	
0	6.2
3	6.2
9	6.2
15	6.0
22	5.8
29	5.6
39	5.6
56	5.2
70	5.2
102	5.2

TABLE II.

The Oxidation of Large Amounts of Sulfur in the Soil in the Presence of Large Amounts of Rock Phosphate.

Period of incubation.	pH value.	Soluble sulfates in 1 gm. of soil.	Citrate-soluble phosphates in 1 gm. of soil.
days		*mg. of SO_4*	*mg. of P*
0	6.2	0.95	2.83
3	6.2	0.96	2.83
9	5.0		
15	3.4	3.60	4.28
22	3.2	20.80	7.13
29	3.0		
39	3.0	30.80	14.76
56	2.2	35.25	20.67
85	2.0		
102	1.8		19.58

Oxidation of Sulfur in Solution by Thiobacillus thiooxidans.

When a proper medium is used, with sulfur as the only source of energy, the pure culture of the organism rapidly oxidizes the sulfur to sulfuric acid. To prevent a rapid change in reaction

buffering substances are used. The course of reaction depends chiefly upon the nature of the buffering agents. When soluble phosphates are used, the curve is more or less continuous; when insoluble phosphates are used the curve has a definite flat portion at a pH of 2.6 to 2.8, the point at which the insoluble phosphates become soluble, and, only after all the phosphate has gone into solution, the curve rises again. When more insoluble phosphate is added at this point, the curve reaction will be kept at the pH of 2.8 to 2.6, till all the insoluble phosphate has disappeared. The medium used for this experiment consisted of sulfur, 10 gm.; $(NH_4)_2\ SO_4$, 2 gm.; $MgSO_4$, 0.5 gm.; $FeSO_4$, 0.01 gm.; KH_2PO_4,

TABLE III.

The Oxidation of Sulfur by Pure Culture of Thiobacillus thiooxidans.

Period of incubation.	No $Ca_3(PO_4)_2$.	0.5 per cent $Ca_3(PO_4)_2$.	Gradual addition of $Ca_3(PO_4)_2$.*
days	*pH*	*pH*	*pH*
0	4.4	5.0	4.4
3	4.4	5.0	4.2
7	3.2	4.4	3.2*
11	2.2	2.8	2.8
17	1.6	2.6	2.6*
23	1.6	2.4	3.2
33	1.4	2.1	3.0
52	1.2	1.8	2.8

* 0.5 gm. of $Ca_3(PO_4)_2$ has been added per 100 cc. of medium.

5 gm.; distilled water to make 1,000 cc. When $Ca_3(PO_4)_2$ is not used, 0.25 gm. of $CaCl_2$ has been added per 1,000 cc. of medium. The medium was placed, in 100 cc. portions, in 250 cc. Erlenmeyer flasks and sterilized on 3 consecutive days in flowing steam. The flasks were then inoculated with a pure culture of *Thiobacillus thiooxidans* by means of a loop, and incubated at 25–27°C. The results are presented in Table III.

It has been pointed out elsewhere (Waksman and Joffe, 1921) that the optimum reaction for the activities of *Thiobacillus thiooxidans* lies at a pH of 3.0 to 4.0. If the reaction of the medium is less acid, the reaction changes in the beginning only very slowly, but, once the optimum is attained, the curve rises rapidly.

A detailed experiment is next reported which will show definitely the relation between the sulfur oxidation, as demonstrated by change in reaction, accumulation of sulfates, and the transformation of phosphates, as shown by the amounts of soluble phosphates and calcium.

A medium containing 2 gm. of $(NH_4)_2SO_4$, 0.5 gm. of $MgSO_4$, 5 gm. of KH_2PO_4, and a trace of $FeSO_4$ per liter, was placed, in 400 cc. portions into fifteen 1 liter Erlenmeyer flasks. 3 gm. of $Ca_3(PO_4)_2$ and 4 gm. of powdered sulfur were added to each flask. The flasks were inoculated with 1 cc. portions of a pure culture of

TABLE IV.

Course of Sulfur Oxidation as Indicated by Change in Reaction and Amount of Soluble Sulfates, Phosphates, and Calcium in the Culture Solution.

Age of culture.	pH	Phosphates in 100 cc. of solution.	Sulfates in 100 cc. of solution.	Calcium in 100 cc. of solution.
		mg. of P	*mg. of SO_4*	*mg. of Ca*
Control.	6.0	123	230	17.4
20 hours.	6.0		230.4	17.44
70 "	5.4			
88 "	4.9	125.26	248.0	24.74
110 "	3.5	123.20	260.15	26.85
134 "	3.0	200.06	322.2	31.0
6.5 days.	2.6	171.64	366.4	64.2
8.5 "	2.6	210.04	498.8	118.8
10.5 "	2.5	255.46	511.4	104.9
13.5 "	2.3	350.00	450.6	101.4
19.5 "	2.1			81.6
34 "	1.3			

Thiobacillus thiooxidans and incubated at 25–27°C. At various intervals, small amounts of the liquid were taken out from four to six flasks, and determinations made of the pH value, of the sulfates, phosphates, and calcium in solution. The results based on the average of four to six determinations, are tabulated in Table IV.

The course of sulfur oxidation is best followed by the change in the hydrogen ion concentration of the medium (pH value). Of course, where there are large amounts of buffering agents or insoluble carbonates or insoluble calcium phosphate, a much larger

amount of sulfur will have to be oxidized to bring about a definite change in the pH value. The amount of sulfur oxidized has been reported in Table IV as sulfates; it may be pointed out here that in practically all cases the sulfur oxidized, as indicated by the amount of residual sulfur in solution, has been almost quantitatively transformed into sulfates. The phosphates and calcium columns in Table IV will be discussed below.

Transformation of Insoluble Phosphate into Soluble Forms.

The reactions involved in the conversion of rock phosphate (insoluble tricalcium phosphate) into soluble forms (di- and monocalcium phosphate and phosphoric acid) by means of acids belong to the type of reactions of heterogeneous systems. The rock phosphate minerals have no definite composition and the products formed are not always definite. In such heterogeneous systems the speed of the reaction is a function of a greater number of variables than in the case of a homogeneous system. According to Kazakov (1913), there are some factors which are common to both systems and these are: (1) concentration of the reacting mass; (2) temperature of the reacting medium; (3) the amount of contact of the reacting substances; (4) the speed of diffusion of the reacting substances; and (5) catalytic agents.

Besides these factors we have others in a heterogeneous system where solid solution phases occur. These are: (1) the size of contact surface;[1] (2) chemical composition of the solid phase; (3) the physical properties of the solid phase; and (4) the influence of formation of a solid phase as a result of the reactions.

The factors; chemical composition of the solid phase, and the physical properties of the solid phase; have a tremendous influence on the speed of the reaction and they are the least known, since the chemical make-up of the rock phosphate is still obscure.

[1] The size of the particles of the rock in the manufacture of acid phosphate has an important influence. Theoretically, all other conditions being equal, the speed of solution of a solid in a liquid is proportional to the contact surface and in circular bodies (as we would suppose in finely powdered rock phosphate) the surface is proportional to the square of the radius; then, particles with a radius of 0.1 mm. will dissolve twenty-five times faster than particles with a radius of 0.5 mm.

The process on the transformation of insoluble phosphates has been the subject of study by a number of investigators in this country and in Europe. We may merely refer to the work of Cameron and Bell (1907) of the Bureau of Soils, of Schucht (1909), Meyer (1905), Stoklasa (1911), Kazakov (1913), and others. According to Kazakov (1913), the scheme of reactions involved in the formation of soluble phosphates are:

No.	H_2SO_4	Resultants obtained.	
		Liquid phases.	Solid phases.
1	When added in excess.	$H_3PO_4 + H_2SO_4 +$ sulfates of Ca, Al, and Fe.	$CaSO_4$
2	Close to optimum.	$H_3PO_4 +$ sulfates of Ca, Al, and Fe.	$CaSO_4$
3	Optimum.	$H_3PO_4 +$ sulfate of Ca + phosphates of Al and Fe.	$CaSO_4$
4	Not enough acid.	$H_3PO_4 +$ sulfate of Ca + phosphates of Ca, Al, and Fe.	$CaSO_4 +$ part of undissolved phosphate.

Before we go into a discussion of the scheme, we shall take up the experimental results of the transformation of the tricalcium phosphate into soluble phosphate through the oxidation of sulfur by *Thiobacillus thiooxidans.*

The culture medium given above has been used, with a slight modification: the KH_2PO_4 was reduced to 1 gm. per liter and 1 gm. of $Ca_3(PO_4)_2$ was added to each flask containing 100 cc. of medium. The medium was sterilized in flowing steam on 3 consecutive days, 30 minutes each day, then the flasks were inoculated with *Thiobacillus thiooxidans* and incubated at 27°C. Only the pH values and water-soluble phosphates (in solution) are reported in Table V and Fig. 1. The results are based on averages of four to six flasks.

The column of soluble sulfates is of extreme interest. At a pH of 2.6, a sudden rise in the amount of soluble phosphates takes place after the soluble sulfates have reached a maximum. This is in accordance with the scheme suggested by Kazakov. Up to the point of pH = 2.6 to 2.7, the liquid phase consists of monocalcium phosphate and gypsum, we therefore have a large amount

of soluble sulfates. However, as soon as more sulfuric acid is formed through the oxidation of sulfur, the monocalcium phosphate is attacked first, since in any reaction the liquid phase comes in first; the products formed are phosphoric acid and gypsum.

$$CaH_4(PO_4)_2 + H_2SO_4 + 2H_2O = CaSO_4 \cdot 2H_2O + 2H_3PO_4$$

The $CaSO_4 \cdot 2H_2O$ is soluble to a certain extent in phosphoric acid, but is forced out from solution because of the fact that the phosphoric acid reacts with the remaining tricalcium phosphate, forming again monocalcium phosphate until the reaction comes to an equilibrium forming gypsum and phosphoric acid. With the

TABLE V.

Course of Sulfur Oxidation and Transformation of Insoluble Phosphates.

Age of culture.	pH	Soluble sulfates in 100 cc.	Soluble phosphates in 100 cc.
		mg. of S	*mg. of P*
Control.	5.4	68.39	45.57
1	5.4	67.64	42.61
2	5.3	69.70	47.20
4	4.6	73.79	55.00
6	3.5		
8	2.6	152.53	103.56
10	2.6	109.7	93.00
12	2.6	78.54	186.30
15	2.4	87.6	207.28

accumulation of the phosphoric acid, more gypsum goes in solution and the soluble sulfates increase again. The continuous increase of the insoluble sulfates after all of the tricalcium phosphate goes in solution is then due to the further oxidation of sulfuric acid.

The column of soluble phosphates also proves the mechanism of the process suggested by Kazakov. Here also we find a gradual increase of the soluble phosphates, since the amounts of sulfuric acid in the early part of the incubation period is small. As soon, however, as the pH reaches 2.6 to 2.7, which is the crucial point of the reaction, the soluble phosphates increase rapidly. Practically all of the tricalcium phosphate goes into solution in 2 days after the crucial point is reached.

The course of conversion of insoluble phosphates in composts of rock phosphate and sulfur has been taken up by Joffe (1922).

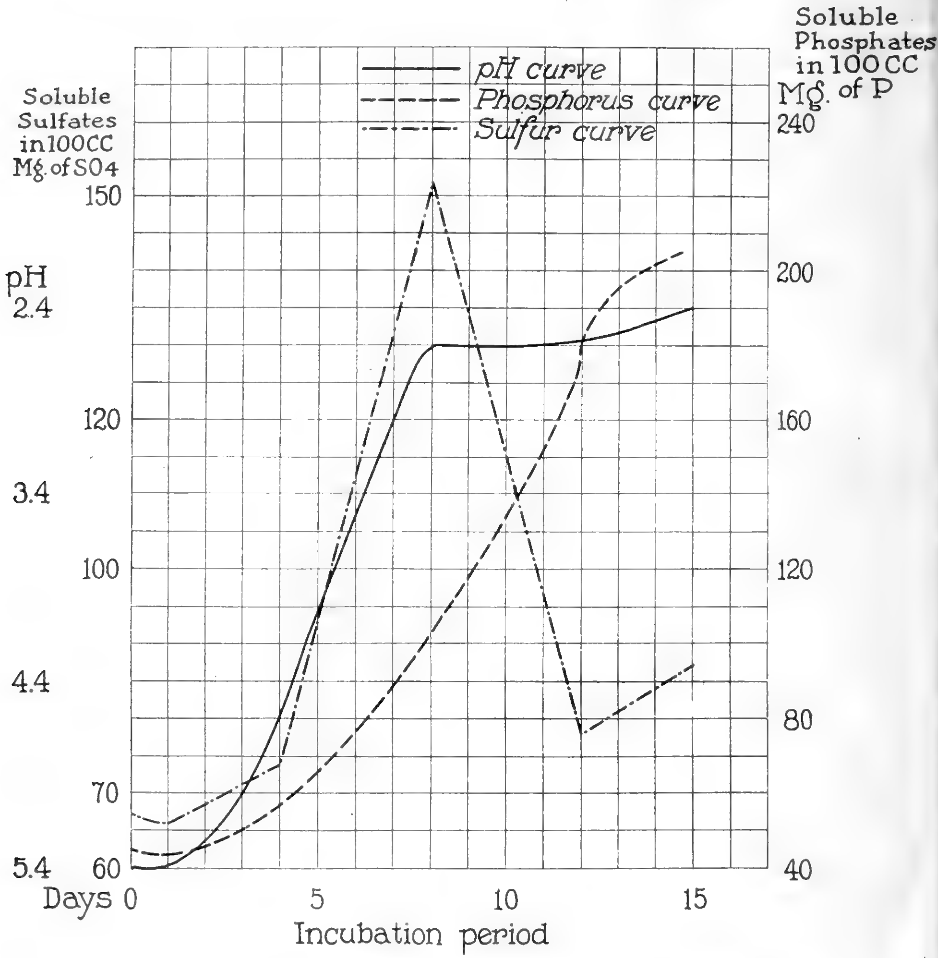

FIG. 1. Change in reaction and increase of soluble sulfates and phosphates by pure culture of *Thiobacillus thiooxidans*.

In this case the reactions are not so apparent, since the indefinite chemical make-up of the raw phosphates introduces a great number of side reactions.

SUMMARY.

1. The curve of sulfur oxidation both in the soil and in solution by pure and impure cultures of *Thiobacillus thiooxidans* is a growth curve.

2. The mechanism of sulfur oxidation to sulfuric acid by *Thiobacillus thiooxidans* obeys the laws of inorganic catalysis.

3. The transformation of insoluble rock phosphate to soluble phosphates by the sulfuric acid formed from the oxidation of sulfur by *Thiobacillus thiooxidans* is similar to the process taking place in inorganic reactions.

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CHANGES IN THE REFRACTIVE INDEX OF THE BLOOD SERUM OF THE ALBINO RAT WITH TEMPERATURE.

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It is a well known fact that changes in the temperature of a solution induce changes in its refractive index (1). In as far as dilute solutions of proteins are concerned it is apparent from the studies of Robertson (2) that these changes are due to changes of the refractive index of the solvent, usually water. This effect on the solvent must be taken into account when measurements of the refractive index are made at different temperatures. Non-recognition of this fact invalidates not a few papers dealing with studies of the refractive index of blood serum. Observations of the temperature variation of this laboratory for over a year have shown an extreme range of 15°C. In view of this fact it became necessary to determine the temperature correction factor for blood serum of the albino rat for use in connection with some refractometric studies now under way.

The sera for examination were obtained from mature albino rats. The animals were etherized and the heart was exposed. A cut was made in the ventricular wall and blood from the beating heart was collected in small test-tubes. These were immediately tightly corked and allowed to stand for 2 or 3 minutes when coagulation was complete. The clot was broken up with a fine glass rod and the serum was separated from the fibrin and corpuscles by centrifuging for ½ hour. At the end of this period the supernatant serum was poured into another small test-tube and again centrifuged for a like period. During the process of centrifuging the tubes were tightly corked to prevent loss of water by evaporation. There resulted from this procedure a clear serum which was transferred to the cell of the refractometer by means of a pipette. After mixing, a portion was taken for the

determination of the water content. The cap of the cell was then lowered into place and the cell was sealed with paraffin having a melting point of 58–60°. This prevented undue loss of water from the sample under examination. The instrument used for these tests was a Pulfrich refractometer made by Carl Zeiss at Jena. Readings were made to tenths of a minute. The instrument was connected with the temperature regulating apparatus described by Reiss (3). Since much of the work reported here was carried on in the winter months the water coming in from the outside contained considerable dissolved air which was liberated on warming and accumulated in the bend of rubber tubing connecting the warming chamber of the cell with the outflow. This interrupted the even flow of water through the instrument and resulted in distressing irregularities of temperature. These were eliminated by inserting into the system a small bottle with a three-hole rubber stopper. Into the central hole there was placed a long glass open tube extending above the level of the tank from which the water is supplied. The other two holes served to connect the bottle with the system. The bottle was placed in the part of the system between the warming coil and the refractometer. With this addition to the apparatus most of the bubbles of air were caught before getting into the refractometer and escaped through the glass tube. When the cell had been sealed a reading was taken of the angle of refraction and of the temperature—usually around 17°—of the enclosed serum. The temperature of the cell and contents was then increased by warming the water circulating through the apparatus. The attempt was made to raise the temperature by steps of 1°. When the new temperature level had been reached and maintained for at least 1 minute another reading was made. This procedure was repeated until the desired maximum temperature had been reached, usually around 35°. The apparatus was then rapidly cooled to approximately the temperature at the beginning of the examination and a final reading was taken. This gave the effect of the heating on the refractive index of the serum constituents. The cell was then opened, a sample of serum removed, and its water content determined. Thus any loss of water from the serum by vaporization during the examination was determined.

As Hatai (4) has shown, the value of the refractive index of the serum of the albino rat varies with the age and to a less degree with the size of the animal. The animals in this series varied somewhat in size although they were all of about the same age. These differences in size resulted in differences in the initial refractive index of the serum. In order that the values might be brought to a common basis for purposes of study the percentage difference between the refractive index of the serum and that of water at the initial temperature of observation was determined. The subsequent observed indices for the serum at the different temperatures were multiplied by this factor, thus making the curve of the change in refractive index with temperature of the serum comparable with the temperature curve of water, the solvent. Any changes in the refractive index due to the influence of temperature on the serum constituents other than water would then be shown by a deviation of the curve for the serum from that for water. The results of the observations reported in this paper were obtained from seventeen sera.

When the calculation noted above had been made it was seen that the sera fell into two groups with respect to their accommodation to the water curve. In the first group there were eight sera. These had been obtained from rats early in the winter. In the second group there were nine series of observations. These had been made during the early spring. The averages of the calculated refractive indices for each degree of temperature were determined for each group and are plotted in Charts 1 and 2, using the curve of the refractive index of water on temperature as the norm.

Chart 1 shows conclusively that in this group (1) of sera the changes in the refractive index with temperature are solely due to the changes in the refractive index of the solvent water. This holds up to a temperature of about 29°. Above this point there is an indication of a tendency for the refractive index of the serum to increase more than does that of water. This curve tends to support the contention of Robertson (2) that when the solvent change is considered the influence of proteins in solution on the refractive index is independent of the temperature between 20 and 40°. In Group 2, however, the curve of which is plotted in

Chart 2, it is seen that the refractive index tends to fall regularly away from that of water with the rise in temperature. The reaction of this group fails to support Robertson's belief mentioned

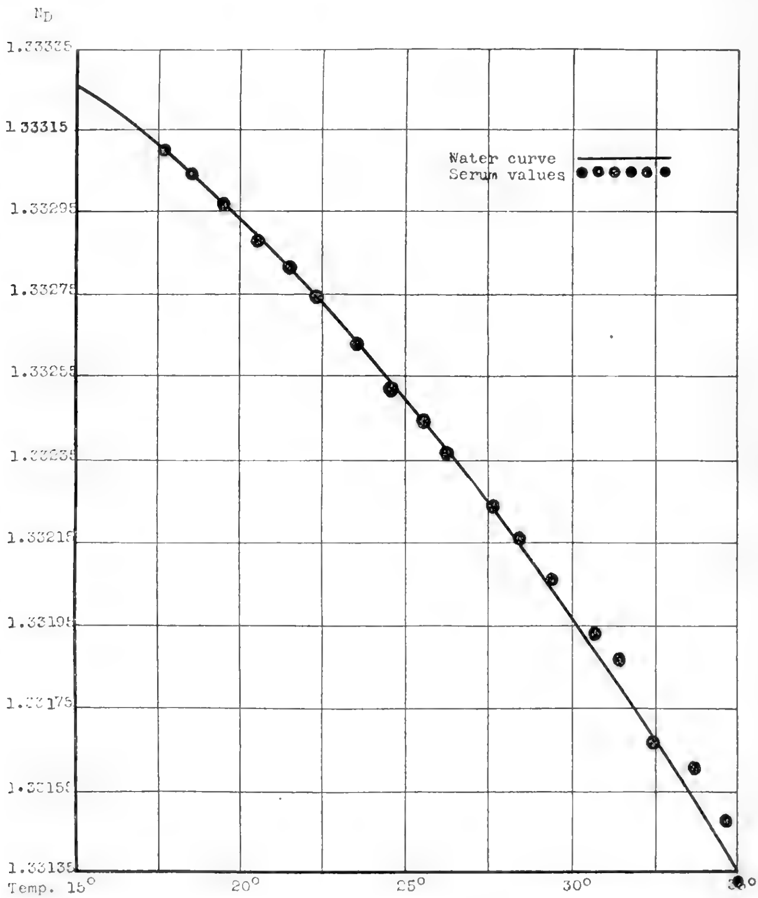

CHART 1. Showing the refractive index of blood serum of the albino rat with increasing temperature. Group 1, winter rats .... Serum values. ——— Water curve.

above. This discrepancy may possibly be explained by the fact that the concentration of protein material in blood serum is considerably above the concentrations used by Robertson in his

temperature studies. This greater concentration may bring into relief differences which in smaller concentrations would be within

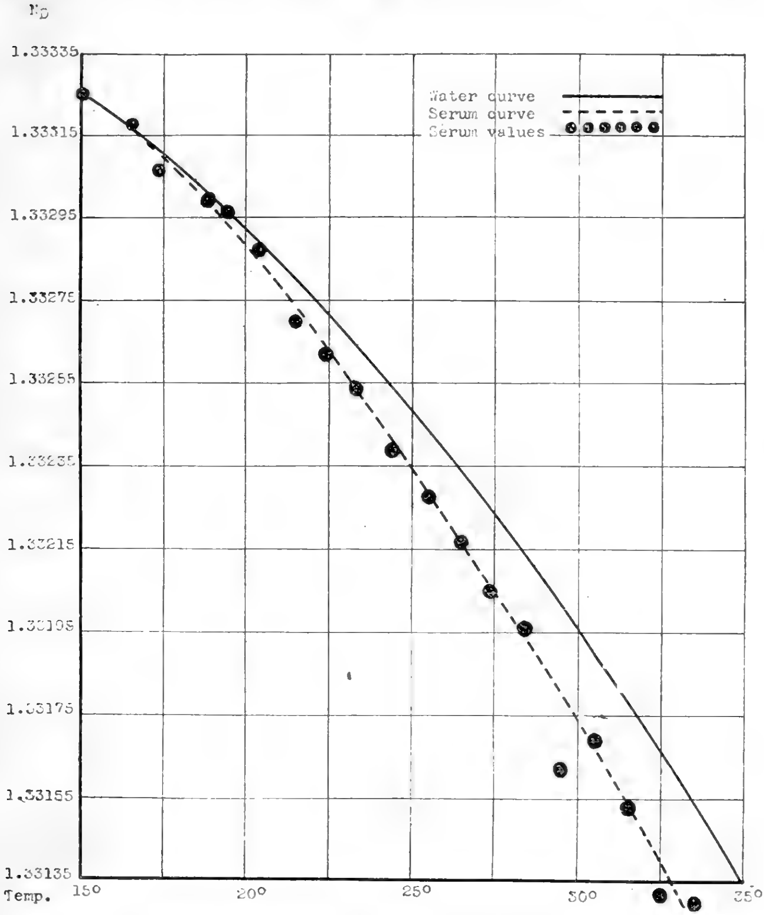

CHART 2. Showing the refractive index of the blood serum of the albino rat with increasing temperature. Group 2, spring rats .... Serum values. ---------- Serum curve. ———— Water curve.

the limit of error of the method of observation and hence unnoticeable. The difference in the temperature effect on the two groups is statistically valid as will be shown presently.

In Table I there is given the mean, standard deviation, and probable error of the mean of the body length, body weight, and water content of the sera in both groups before and after the refractometric examination. The table shows that none of these are factors in the difference in behavior of the sera towards changes in temperature. Nor can the difference be attributed to a general difference in the state of digestion and absorption, since at least 18 hours regularly intervened between the last feeding and the time of the taking of the blood. The only factor at present indicated is a possible seasonal variation, since the determinations of the first group were made on winter rats and those of the second group on spring rats. Although we would not care to stress this point, when the fact that in the spring there is an increased sexual activity in the rat is correlated with the

TABLE I.

	Group 1.			Group 2.		
	Mean.	Standard deviation.	Probable error of mean.	Mean.	Standard deviation.	Probable error of mean.
Body length, *mm*...........	188	12.5	3.4	184	15.1	3.6
Body weight, *gm*...........	183	26.6	7.3	180	50.1	11.9
Water, before, *per cent*.....	92.2	0.51	0.13	92.2	0.25	0.06
Water, after, *per cent*.......	92.0	0.45	0.11	92.0	0.45	0.10

observation of Hatai (4) that irregularities in the course of the curve of the refractive index of the serum of the albino rat on age occur around the period of puberty, it would appear as if an interpretation based on such a seasonal modification is strengthened.

Whatever the determining factor may be, it is evident that in some sera certain constituents other than the solvent water are so influenced by temperature changes that they give rise to changes in the refractive index entirely apart from those produced by the solvent.

Robertson (5) has stated that the change in the refractive index of a solvent is a function of the size of the molecules of the solute. This, of course, refers to conditions at uniform temperature. It is not improbable that the state of the colloidal equilib-

rium existing in those sera showing regular deviations from the water curve with rising temperature may be relatively unstable and that as a result of the changes in temperature, changes in the degree of dispersion of the colloidal constituents take place. Such an assumption is supported by Robertson's (6) observation that as protein solutions approach the point of coagulation there occurs a decrease in the refractive index. It should be noted in this connection that this change is not rapidly reversible since when the serum is cooled to the point where the initial reading was taken, the value of the refractive index is usually somewhat greater than it was at the beginning of the examination.

Turning now to the practical application of these results, there is given in Table II, the mean, standard deviation, and probable error of the mean of the change in reading of the angle of refraction for 1° of temperature for the two groups. It is seen that in the first group each rise of 1° is accompanied by a corresponding increase of approximately 1 minute in the angle of refraction. In the second group the increase of the angle of refraction for each degree of temperature is 1.4 minutes, a somewhat greater value. This difference between the two groups is statistically valid and substantiates the curve in Chart 2.

TABLE II.

Statistical Values of the Angles in Minutes of Refraction of the Two Groups.

	Group 1.	Group 2.
	minutes	*minutes*
Mean	1.08	1.41
Standard deviation	0.23	0.36
Probable error of mean	0.02	0.03

Notwithstanding the difference, an inspection of the index of variability shows that for all practical purposes the mean of the change in the angle of refraction of the two groups—1.25 minutes—for each degree of temperature is an acceptable factor and gives results accurate within plus or minus half a minute of refraction. This can be used for correction of the observed readings. Taking 20° as the standard temperature, when a refractometric reading is made of blood serum at a temperature above

this value, 1.25 times the difference between 20° and the observed temperature should be subtracted from the observed reading. If the temperature is between 17.5 and 20° the difference times the factor is added. The following formulas are simple expressions of this relation; $I=i-1.25(t-20°)$ and $I=i+1.25(20°-t)$ where I is the corrected angle of refraction; i the observed angle of refraction; and t the observed temperature.

SUMMARY AND CONCLUSIONS.

A study of the changes in the refractive index of the blood serum of the albino rat with rising temperature showed that two types can be distinguished according to the nature of the response. In the first type the changes in the refractive index coincide with those of the solvent water and can be attributed to this serum constituent. In the second type the curve of the change of refraction with rising temperature falls away from that of water. This demonstrates a participation in the response of serum constituents other than the solvent water. The causes of this difference are unknown, although there is a possibility that a seasonal variation may be a determinant. It is certain that in this series the factors of body length, body weight, age, and water content of the serum both before and after the experiment, and previous state of digestion and absorption are not the causes of the difference between the two groups.

The correction for the reduction of the observed angle of refraction to the common base at 20° when readings are taken at different temperatures is obtained by use of formulas given in the text. These formulas hold for temperatures between 17.5 and 35°C.

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COLORIMETRIC DETERMINATION OF URIC ACID.

ESTIMATION OF 0.03 TO 0.5 MG. QUANTITIES BY A NEW METHOD.

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Determinations of uric acid, which depend upon the direct weighing of the substance as such or upon an estimation of the nitrogen content of an insoluble salt, have been largely replaced by methods which quantitatively measure its oxidation. Any of these procedures, whether volumetric or colorimetric, are useful in proportion to their success in (*a*) separating the uric acid from other substances which might give a value in the oxidation reaction and in (*b*) making the actual conditions of the reaction as specific as possible for uric acid.

Volumetric methods, in most cases, have not been sufficiently sensitive for satisfactory application to less uric acid than is found in 100 cc. of urine. Also the means for preliminary precipitation of uric acid made use of in these methods were crude. One of us[1] determined conditions for a complete precipitation of uric acid in any amount with zinc salts, and found conditions under which permanganate oxidation could be used successfully on such quantities of uric acid as are found in 5 cc. of urine.[2] The much smaller amount of uric acid found in blood is below the limit of accuracy which it is possible to attain with the zinc precipitation-permanganate oxidation procedure.

Folin and Macallum[3] recognized the need of separation of uric acid from interfering substances and attempted the removal of polyphenols from urine residues before oxidizing uric acid with

[1] Morris, J. L., *J. Biol. Chem.*, 1916, xxv, 205.

[2] Morris, J. L., *J. Biol. Chem.*, 1919, xxxvii, 231.

[3] Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912–13, xiii, 363

alkali phosphotungstate. Folin and Denis[4] improved this separation by their adaptation of the Salkowski[5] precipitation of uric acid to precede the phosphotungstate oxidation. The method described in this paper makes use of the zinc salt separation of uric acid which has proved very satisfactory in our hands and determines the uric acid so separated by a new colorimetric procedure which possesses the double advantage of greater specificity and greater color obtainable for unit weight of uric acid. With slight variations it is equally applicable to urine and blood. In the latter case we have removed the proteins by the tungstic acid precipitation of Folin and Wu[6] and found the procedure satisfactory for our purpose.

The results which we have obtained with the new method are, we believe, more dependable and uniform than the usual ones obtained with any of the procedures based upon silver precipitation. One cause for irregularities in the results obtained by means of the silver methods is inherent in the metal used. Silver solutions, even when protected from light, soon become clouded with, and eventually precipitate, a form of "reduced silver." The acid silver lactate solution used by Folin and Wu,[6] is noticeably cloudy soon after preparation and develops a heavy precipitate on standing. The ammoniacal silver magnesium solution of Benedict and Hitchcock[7] also forms a scum around the neck of bottles, spouts of dropping bottles, etc., which occasionally contaminates the reagent added and thus gives erroneous results. This "reduced silver," when taken up in sodium cyanide and the mixture made alkaline with sodium carbonate and phosphotungstic acid reagent added, gives a deep blue color even when taken in small amounts. (The color can also be developed by reversing the order of these reagents.) This possibility of the presence of such a substance is sufficient to cast doubt upon any determination which makes use of a silver precipitation. It is evident that the urine procedure of Folin and Wu[8] is particularly

[4] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 469.

[5] Salkowski, E., *Virchows Arch. path. Anat.*, 1870, lii, 58. Ludwig, E., *Wien. med. Jahrb.*, 1884.

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

[7] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 459.

open to criticism on this point for the reason that the entire precipitate is dissolved in sodium cyanide previous to the development of color. The extraction step of their blood procedure to a large extent eliminates this error. Myers'[9] suggestion in regard to this method that cyanide be added before the second centrifuging can hardly be accepted as an improvement when considered in the light of these facts. We have found that the reliability of these methods is greatly increased when care is taken to use only perfectly clear silver reagents.

Another possible source of error in the Folin and Wu procedure is a common impurity found in many of the best grades of sodium sulfite we could obtain. Three out of four lots of this salt, from as many sources, gave a reaction with the uric acid reagents. The color which developed in each instance was sufficient to introduce a considerable error if the sulfite had been used in the amount prescribed for the determination.

Neither silver nor sulfite is used in the new method described in this paper. The new method requires the use of no metal which may exist in a reduced form or reagent which is likely to contain any interfering impurity. Fortunately, in addition to their adaptability to the purpose, there is the additional advantage that all the reagents used are relatively inexpensive.

The use of potassium cyanide suggested by Benedict and Hitchcock[7] for tying up the silver greatly improved the earlier Folin-Denis[4] method. We successfully applied cyanide for the purpose of forming a double radical with zinc and found it useful as well in two other particulars. Used in larger amounts its alkalinity is sufficient for the complete and rapid development of the color when the actual molecular concentration of alkali is still less than one-third that used in the Benedict-Hitchcock procedure. The second advantage in the use of cyanide is the very marked increase of color which is obtained from a given amount of uric acid.

That this increase is due to the uric acid reduction of phosphotungstate, while the cyanide only accelerates the reaction, is shown by results of the kind typified in Table I. The negative result of Flask 10 shows that the cyanide will not act directly on the phosphotungstate. The 18 per cent increase of color in

[9] Myers, V. C., *J. Lab. and Clin. Med.*, 1919–20, v, 499.

TABLE I.

Effect of Sodium Cyanide upon Phosphotungstate Oxidation of Uric Acid.

Flask No.	Uric acid.	5 per cent sodium cyanide.	Colorimeter reading.	Value found.
	mg.	*cc.*	*mm.*	*mg.*
1	0.25	None.	30.0	1.00 × 0.25
2	0.25	0.05	25.5	1.18 × 0.25
3	0.25	0.25	21.1	1.42 × 0.25
4	0.25	0.50	20.4	1.47 × 0.25
5	0.25	1.00	19.2	1.56 × 0.25
6	0.25	2.00	16.1	1.86 × 0.25
7	0.25	4.00	14.1	2.13 × 0.25
8	0.25	8.00	12.5	2.40 × 0.25
9	0.25	12.00	12.2	2.46 × 0.25
10	None.	10.00	No color.	None.

Color developed in each case by addition of 1 cc. of phosphotungstic acid reagent and 10 cc. of 20 per cent sodium carbonate. All made up to 50 cc. volume after 10 minutes and compared with No. 1.

TABLE II.

Effect of Sodium Cyanide upon Arsenotungstate Oxidation of Uric Acid.

Flask No.	Uric acid.	10 per cent sodium cyanide.	Colorimeter reading.	Value found.
	mg.	*cc.*	*mm.*	*mg.*
1	0.4	1.0	148.0	0.054
2	0.4	1.5	52.0	0.156
3	0.4	2.0	34.4	0.236
4	0.4	2.5	30.0	0.266
5	0.4	3.0	27.1	0.287
6	0.4	3.5	25.9	0.309
7	0.4	4.0	24.7	0.324
8	0.4	4.5	23.5	0.340
9	0.4	5.0	21.3	0 376
10	0.4	7.5	20.2	0.396
11*	0.4	10.0	20.0	0 400
12	0.4	15.0	20.0	0 400
13	None.	10.0	No color.	None.

Color developed by addition of 2 cc. of arsenotungstic uric acid reagent and the amount of cyanide indicated for each flask.

* No. 11 was used as a standard and the others compared with it.

Flask 2, due to 1 drop of cyanide, recalls an increase of the same amount which Benedict and Hitchcock observed under similar conditions (2 drops of cyanide and 15 cc. of carbonate in a 50 cc. flask). Flasks 8 and 9, with 50 per cent more cyanide in the latter, showed nearly a constant value with two and a half times as much color as Flask 1. Obviously the cyanide causes displacement of the equilibrium so as to approach the maximum amount of the blue compound for these conditions. Attempts to determine the full extent of this effect met with little success at first for the decrease of carbonate and increase of cyanide was required to accomplish further deepening of the color, and this change in the alkalies caused precipitation before the time for development of color had passed. Also we found that a certain amount of carbonate was necessary to prevent the blue color which otherwise develops when phosphotungstic acid reagent is made alkaline with sodium cyanide.

We had previously made many conjugated tungstic acids, substituting analogous acids for phosphoric, concerning which we expect to make a more extended report soon. Upon trial we found that one of these, arseno-18-tungstic acid, in addition to other desirable properties, gave absolutely no color with sodium cyanide even when no other alkali than the cyanide was present, and did not precipitate in the presence of large amounts of cyanide. By its use we made further observations of the effect of cyanide upon the oxidation of uric acid by tungsten compounds and secured the results recorded in Table II. It will be noted that the amount of uric acid used in each flask was 0.4 mg., for our experience had shown that the color obtained from that quantity was of a desirable depth for colorimetric comparisons. It is also apparent that the color in Flasks 1 to 10 increased with additional quantities of cyanide, the increments being progressively smaller. In Flasks 11 and 12 there was practically no increment, though the quantities of cyanide were, respectively, 133 and 200 per cent of that in Flask 10. Evidently the conditions had been reached by which the oxidation of uric acid by a tungsten compound was complete.

The determination of relations between the amount of color obtainable by the new arsenotungstate-cyanide method and the former phosphotungstate-carbonate procedures could not be abso-

lute, owing to the great difference in the concentrations of the reaction liquids. Approximate comparisons show that the new uric acid method gives 3.3 times the color of the Folin-Macallum-Denis method, 2.8 times that of the Benedict-Hitchcock procedure, and 2.5 times that of the Folin-Wu method. There is, of course, a great mechanical advantage in being able to get three times as much color from the very limited amount of uric acid in blood and other body fluids. In addition, there is greater satisfaction in the realization that the deeper color made possible by the new method represents a truer value of the uric acid present in that it is the result of a complete chemical action and not subject to the disturbing variations which may occur in procedures which depend upon artificially maintained equilibria.

Finally, the use of sodium cyanide in the manner described in this paper possesses another advantage in the selective application of its driving power. How exclusive this selective action of cyanide, whether there are other substances present in body fluids upon which it will act, and what the nature of the driving power may be are questions now being investigated further in this laboratory.

Method.

Reagents.

Preparation of Arseno-18-tungstic Acid Solution.—Boil a mixture of 100 gm. of hydrated sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), 125 gm. of arsenic acid anhydride (As_2O_5), and 650 cc. of water for 2 to 4 hours in a flask. If the reagent so formed has a blue or green color after it has boiled the required time, it should be decolorized by boiling with sufficient bromine water to make the color a clear yellow or yellowish brown.[10] After boiling off any excess bromine add distilled water to make the volume 1 liter. The arsenotungstic acid reagent so prepared is a somewhat lighter color than the phosphotungstic acid reagent.

Other Reagents Required.—2.5 per cent zinc chloride solution; 10 per cent sodium carbonate solution (if monohydrated sodium carbonate is used, allowance must be made for the water of crystallization); 10 per cent hydrochloric acid solution; 10 per cent sodium cyanide solution; standard uric acid solution (phosphate solution of Benedict-Hitchcock).[7]

For Removal of Proteins.[6]—10 per cent sodium tungstate; $\frac{2}{3}$ N sulfuric acid, within 5 per cent by titration; solid potassium oxalate.

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[10] Decolorization in this way is desirable for any conjugated tungstic acid which is to be used for colorimetric work. A dark blue or green reagent (either phospho- or arsenotungstic acid) introduces a very noticeable error when used where the color to be read is light.

Determination.

The method is essentially the same when used in uric acid solutions of such different concentration as urine and blood. Convenient quantities of reagents and choice of volumetric flasks which facilitate colorimetric comparison are the principal points of difference in the procedures described.

Procedure as Used in Urine.—Pipette 1 cc. of urine into a 50 cc. centrifuge tube and dilute with distilled water to about 40 cc. Add 1 cc. of 2.5 per cent zinc chloride and mix with a stirring rod. Add 1.0 cc. of 10 per cent sodium carbonate which should make the solution alkaline to litmus and stir thoroughly. Centrifuge for about 2 minutes, drain off, and discard the supernatant liquid. Dissolve the residue, with stirring, in 3 or 4 drops of 10 per cent hydrochloric acid, dilute with 5 cc. of water, add 10 cc. of 10 per cent sodium cyanide, and transfer quantitatively to a 100 cc. volumetric flask, and dilute to about 60 cc. If 1 cc. of urine contains more than 0.5 mg. of uric acid the amount of cyanide should be doubled (20 cc.) and a 200 cc. flask used. In this case dilute to about 120 cc. To prepare a standard containing 0.2 mg. in 50 cc. pipette 1 cc. of the phosphate standard solution into a 50 cc. volumetric flask and 25 to 30 cc. of distilled water and 5 cc. of 10 per cent sodium cyanide. Develop the color in both by addition of the arseno-18-tungstic acid reagent, 1 cc. to the standard (50 cc. flask), 2 cc. to the unknown if in 100 cc. flask or 4 cc. if in the 200 cc. flask. Shake, dilute to volume, let stand 2 or 3 minutes, and compare in the colorimeter. The color develops with such rapidity that the time interval indicated is sufficient if the standard and the unknown are made simultaneously. If, for any reason, they are not so prepared, it is best to allow 10 minutes to elapse before making the color comparison.

Procedure as Used in Blood.—Collect oxalated blood in the usual manner, drawing the blood from a vein into a weighed flask containing 2 mg. of potassium oxalate for each cubic centimeter of the sample taken. After determining the amount of blood by weight, pour it into seven times its volume of distilled water, add 1 volume of 10 per cent sodium tungstate solution and then, while shaking, run in slowly 1 volume of $\frac{2}{3}$ N sulfuric acid. Shake for several minutes and filter (precipitation method of Folin and Wu[6]). Pipette 25 cc. of the clear filtrate (corresponding to 2.5 cc. of blood) into a 50 cc. centrifuge tube and dilute with distilled water to about 40 cc. Add 1 cc. of 2.5 per cent zinc chloride and mix with a stirring rod. Add 1.0 cc. of 10 per cent sodium carbonate to make just alkaline to litmus and stir thoroughly. Centrifuge for about 2 minutes, drain off and discard the supernatant liquid. Dissolve the residue with stirring in 3 or 4 drops of 10 per cent hydrochloric acid, dilute with 5 cc. of water, and add 2.5 cc. of 10 per cent sodium cyanide and transfer quantitatively to a 25 cc. volumetric flask. Prepare two standards containing 0.1 and 0.2 mg. in 50 cc., by pipetting 0.5 and 1 cc. of the phosphate standard solution into two 50 cc. volumetric flasks. Add about 30 cc. of distilled

water and 5 cc. of 10 per cent sodium cyanide to each. Develop the color by the addition of the arseno-18-tungstic acid reagent, 0.5 and 1 cc. respectively into the unknown and standards. If the color has been developed simutaneously, shake, dilute to volume, let stand a minute or two, and compare in the colorimeter; if not, the same lapse of time should be allowed as indicated in the case of urine.

Both procedures are adapted to the quantities of uric acid found in the largest number of urine and blood samples analyzed by us. In a very few cases we found it advantageous to choose volumetric flasks of a larger or smaller size to contain the unknown. This may be done with good results if the concentrations of arseno-18-tungstic acid and sodium cyanide are kept comparable. For this purpose the following simple rule must be observed:

100 cc. flask contains 10 cc. of 10 per cent sodium cyanide and 2 cc. of arseno-18-tungstic acid reagent; 50 cc. flask contains 5 cc. of 10 per cent sodium cyanide and 1 cc. of arseno-18-tungstic acid reagent; 25 cc. flask contains 2.5 cc. of 10 per cent sodium cyanide and 0.5 cc. of arseno-18-tungstic acid reagent.

TABLE III.

Comparative Estimations of Uric Acid in Urine.

Urine.	Benedict-Hitchcock method.	Folin-Wu method.	New method.
	mg.	*mg.*	*mg.*
1	390	375	388
2	752	625	702
3	514	492	533
4	547	498	508

Table III presents comparative uric acid results for several urine specimens. In their analyses we used the method described here, the Folin-Wu method, and the Benedict-Hitchcock procedure. Precaution to use only the clearest possible, silver reagents had the effect of practically eliminating irregularities due to reduced silver. In spite of similar precautions in work with blood specimens there were marked irregularities. Upon undertaking the work of their explanation we were led into various problems connected with the chemistry of the methods and the chemical nature of the uric acid present in blood. Some of the results obtained appear in the following paper.[11]

[11] Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, l, 65.

SUMMARY.

Combination of zinc precipitation with a new colorimetric method has made possible the estimation of very small quantities of uric acid. Arseno-18-tungstate proves a great improvement over phospho-18-tungstate of earlier methods. Sodium cyanide is used as the only alkali for development of color and serves to bring about the complete oxidation of uric acid. In comparison with the amount of color obtained in the methods which depend upon oxidation to a point of equilibrium, the new conditions of complete oxidation permit the development of three times as much color per unit weight of uric acid. The same conditions that bring about completion of the reaction are also responsible for greater speed in reaching the maximum color and in a very marked permanency of the color. The use of cyanide as alkali practically eliminates the precipitation of various compounds in the colored liquid which, next to fading, was the most serious difficulty accompanying the use of carbonate.

Precipitation of uric acid with zinc salts lends itself just as well to the subsequent formation of a double radical with cyanide as in the case with silver methods. In addition there is excluded all possibility of a reduced metal giving erroneous results in the later oxidation reaction.

Finally, the number of reagents required is small, they are easily prepared and the use of inexpensive zinc chloride instead of expensive silver salts makes the determination much more desirable, especially where many analyses are run, as in medical classes and extended research work on purines.

STUDIES ON THE URIC ACID OF HUMAN BLOOD.

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Uric acid estimations have probably been subject to more irregularities and losses than attend the analysis of other well known biological products. Explanations of these irregularities have usually been based upon its very slight solubility, the unusual ease and variety of its oxidation reactions or the more vague property of different forms in which it has been supposed to exist in body fluids. The new method of uric acid analysis described by the authors in the foregoing paper,[1] while characterized by extraordinary agreement between successive determinations in urine and blood, differed in quite an irregular manner when its values for blood were compared with those obtained by the method of Folin and Wu.[2] The present paper sets forth some of the results of our efforts to find an explanation for the apparent discrepancies. In addition to evidence which casts considerable doubt upon the accuracy of uric acid data obtained through the use of earlier methods, we have made observations which can be explained only by the existence of uric acid in more than one form in human blood.

Differences in form of uric acid, or more correctly urates, have been used as a basis for several hypotheses concerned with the physiology and pathology of purine metabolism. One of the more definite ideas of these differences is that which pictures uric acid present in various stages of change according to the effect of blood conditions upon its property of keto-enol isomerism. Gudzent[3] first discovered that uric acid forms two series of primary

[1] Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, l, 55.

[2] Folin, O. and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

[3] Gudzent, G., *Z. physiol. Chem.*, 1909, lx, 38.

urates, differing in stability and solubility. They were named the lactam and lactim forms. Shifting the point of equilibrium between these tautomeric forms might conceivably take place under changing blood conditions. The resulting change of point of saturation has been urged as an explanation of many clinical symptoms, such as the deposition of urates in cartilages of gout patients, etc.

Another idea of the difference in form of uric acid is that some part of the whole amount is combined with another substance or substances. Minkowski[4] advanced the hypothesis that uric acid may be combined with nucleic acid in the body tissues and suggested that this combined uric acid might regulate the chemical relationships of free uric acid. The basis for this conclusion was the observation that the precipitation of a uric acid solution by acetic acid or ammoniacal silver magnesium solution is prevented by the addition of nucleic acid. No experimental evidence has been advanced that combined uric acid exists in any part of the human body. Benedict[5] several years ago found combined uric acid in mixed beef blood, in quantity many times greater than the free uric acid in the same blood. A year later Benedict[6] made the following statement, "with the exception of man, all mammals probably have two forms of uric acid in the blood. In the case of human blood the data so far available are not conclusive. It is quite probable that here, too, uric acid exists in the blood in at least two forms but they are quite unlike the forms present in ox blood." Davis and Benedict[7] recently reported the isolation of a crystalline substance from beef blood which they identified as a ribose-uric acid compound. The data presented later in this paper furnish the first positive experimental proof that human blood contains uric acid in at least two forms.

We analyzed many samples of blood and serum by the Folin-Wu method and by the new method in order to compare the values

[4] Minkowski, O., Die Gicht, in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1903, vii, pt. 2, 189–190.

[5] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 633.

[6] Benedict, S. R., *J. Lab. and Clin. Med.*, 1916–17, ii, 1.

[7] Davis, A. R., and Benedict, S. R., *J. Biol. Chem.*, 1921, xlvi, p. v.

so obtained. In all bloods the Folin-Wu method of protein precipitation was used, the filtrate in each case serving as a common source of the samples taken for both methods of analysis. Recognizing the danger of abnormal results in the Folin-Wu method due to the possible presence of "reduced silver" every precaution was taken to assure the use of clear silver lactate solution.

TABLE I.

Uric Acid Estimation in Blood. Comparison of Methods.

Specimen.	Folin-Wu method.	New method.
Group 1. Examples of blood and serum which show marked irregularities.		
	mg.	*mg.*
Blood 1, C. B. I.	4.1	4.9
" 2, G. F.	2.7	3.4
" 3, A. M.	1.8	2.3
" 4, C. M.	4.4	5.5
" 5, J. K.	1.5	2.3
" 6, J. D. III.	1.7	2.7
" 7, C. B. II.	2.7	3.4
" 8, G. C.	2.7	4.8
" 9, J. Dh. II.	1.9	3.0
Mixed serum A.	1.4	3.1
" " B.	1.6	2.5
Group 2. Examples of blood which show little or no irregularity.		
Blood 1, G. M.	7.7	7.9
" 2, J. W. II.	3.5	3.2
" 3, J. H.	3.4	3.4
" 4, I. C.	1.8	1.6
" 5, C. B. II.	3.2	3.2
" 6, D. H.	2.3	2.3
" 7, J. D. I.	1.8	2.1

In addition, suspicious variations between duplicates, were immediately followed by check determinations. In this way the Folin-Wu values which are recorded in this paper represent the highest accuracy which can be obtained by the method. Even after these unusual precautions there were puzzling irregularities in the agreement of the two series of results. Typical examples of the comparative blood analyses are presented in Table I.

It is apparent from the figures tabulated that the new method gives values for blood which range from those which are essentially the same (Group 2, Bloods 1 to 7) to those (Group 1, Bloods 1 to 9) which are higher than the corresponding Folin-Wu values. The largest increase in value shown in the bloods is 75 per cent. In the case of the mixed serums, A and B, the results are similar but the amounts of increase range from 60 to 120 per cent. This high range of increase is characteristic of all mixed serums we have had under observation. We made repeated attempts to lower the results of the new method to the level of the Folin-Wu method. We could neither accomplish such a decrease nor find evidence of the presence of any substance which gave an added color value. The specific nature of the new cyanide-arsenotungstic reaction (in its driving effect upon uric acid to the exclusion of any other substance so far investigated) was good evidence that the higher value is due to a more complete measurement of the uric acid present. Attempts were then made to increase the Folin-Wu values. None of these was successful until we added potassium oxalate. Though smaller amounts of oxalate have the effect of elevating the value obtained with the Folin-Wu method, we found it desirable to use an excess sufficient for all possible variations in blood specimens. Therefore, we added 100 mg. of potassium oxalate (measured as 10 cc. of a 1 per cent solution) to each 20 cc. quantity of filtrate before beginning the determination. The presence of the oxalate increased the results of the determination as shown in Table II.

The recorded results obviously fall again into two groups. The values of the Folin-Wu determinations are markedly increased in the first group of seven blood samples and two mixed serums while there is no increase or a very slight increase in the second group of six bloods. Evidently the value found by the new method determines not only the question of whether an increase will result from the use of potassium oxalate but constitutes the approximate limit of the increase when present. With minor differences of the order of variation between duplicates, the figures secured by means of the modified Folin-Wu method (potassium oxalate preceding the Folin-Wu procedure) are the same as those obtained by the new method. This observation is peculiarly significant when considered in connection with the

fact that the presence of oxalate had little or no effect upon the analysis of those blood specimens (entered as Nos. 1 to 6 of Group 2, Table II) for which both methods gave essentially the same values. The explanation of the irregularities was thus shown to be less a matter of method weakness and more a matter of character of the content of individual bloods.

TABLE II.

Effect of Potassium Oxalate upon Blood Uric Acid Values Obtained by the Folin-Wu Method.

Specimen.	Folin-Wu method.	Folin-Wu method after addition of $K_2C_2O_4$.	New method.
Group 1. Examples of blood and serum which show a marked increase.			
	mg.	*mg.*	*mg.*
Blood 1, C. B. I	4.1	5.2	4.9
" 2, G. F	2.7	3.5	3.4
" 3, A. M	1.8	2.3	2.3
" 4, C. M	4.4	5.4	5.5
" 5, J. K	1.5	2.7	2.3
" 6, J. D. III	1.7	2.2	2.7
" 7, C. B. II	2.7	3.2	3.4
Mixed serum A	1.4	4.3	3.2
" " B	1.6	3.3	2.5
Group 2. Examples of blood which show little or no increase.			
Blood 1, G. M	7.7	8.0	7.9
" 2, J. W. II	3.2	3.3	3.2
" 3, J. H	3.4	3.7	3.4
" 4, I. C	1.8	1.6	1.6
" 5, C. B. II	3.2	3.6	3.2
" 6, D. H	2.4	2.3	2.2

We undertook an investigation of different blood specimens in the hope of finding what chemical difference exists that determines for each specimen whether the values given by the silver and zinc methods are to agree or disagree and a corresponding agreement or disagreement between the values by the former method in its original form and as modified by the addition of oxalate. The very limited quantity of blood in each case hindered

progress of the investigation as did also the impossibility of judging before analysis whether each new specimen would show agreement or disagreement between the methods. The use of other than human blood was inadvisable in view of the radical difference in form and quantity recognized by Benedict as characterizing the blood uric acid of different species. Analysis of mixed serums (from many blood specimens drawn for routine Wassermann tests and found negative) consistently showed the new method value higher than the Folin-Wu value. Furthermore, the percentage increase was about twice as great as in the case of whole blood specimens. Evidently serum was by nature and quantity availability the best material for use in identifying the character of the substance responsible for the divergent uric acid values. Serum was saved over a period of weeks. The proteins were precipitated from each day's quantity of serum by the tungstic acid method. The filtrates of successive days were poured together until there was a volume of 5 liters. Several such lots were investigated after each was analyzed by the three procedures. The two sets of results presented in the foregoing tables are typical analyses of mixed serum.

As a result of many observations on the mixed serums we were convinced that we were dealing with more than one form of uric acid. We attempted the separation of uric acid as such for evidence supporting the analytical data. 1,000 cc. of mixed serum were precipitated in 50 cc. portions by the zinc procedure. After centrifuging and pouring off the mother liquid the combined precipitates were dissolved in 10 per cent acetic acid, then 100 cc. of water and about 0.5 gm. of bismuth carbonate added. Hydrogen sulfide was bubbled through the solution until it was saturated; it was then heated to the boiling point and filtered. The filtrate was evaporated to small quantity and then heated to dryness on the water bath. After dissolving in 25 cc. of hot water and transferring to a 50 cc. centrifuge tube, the uric acid was again precipitated by the addition of 4 cc. of the ammoniacal silver magnesium reagent of Benedict and Hitchcock and separated by centrifuging.[8] To the precipitate were added 5 cc.

[8] The second precipitation (as a silver compound) and subsequent separation of uric acid as such substantially follows the method used by Benedict in identifying uric acid in beef blood (Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 637).

of water and 5 cc. of saturated hydrogen sulfide solution (freshly prepared). After mixing thoroughly the silver sulfide was removed by filtration and the hydrogen sulfide from the filtrate by evaporation to dryness on a water bath. The residue was taken up in successive small portions of boiling water and transferred to a weighed 15 cc. centrifuge tube. The volume was then about 5 cc. 1 cc. of glacial acetic acid was added and the tube set aside. 5 days later the mother liquor was poured off and the characteristic crystalline precipitate of uric acid washed first with water and then with alcohol. After drying, the tube was weighed. In addition to the weight and crystalline form further evidence of the nature of the precipitate was obtained by colorimetric analysis. The characteristic acid precipitation was checked by the data so secured. The results are presented in Table III and with them are recorded, for comparison, the values obtained in the similar treatment of a standard uric acid solution, which contained 3 mg. and was diluted to a liter before precipitation.

Reference to the table shows in the case of each serum that, after the laborious process of removing uric acid from solution twice by formation of two different salts, the amount found present by analysis of the final crystalline product is greater than the Folin-Wu method originally indicated. (Serum A shows 2.2 mg. against 1.4 mg. and Serum B shows 2.0 mg. against 1.6 mg.) On the other hand, the amount of uric acid which could be similarly recovered from the standard solution is much less than was originally present (1.2 mg. from 3.0 mg.). The latter yield is as large as might be expected after the tedious steps of the double precipitation, the subsequent removal of the metallic sulfides, filtrations, evaporations, etc. If the loss in the case of the serum filtrates was actually comparable to that of the uric acid solution, and we should not expect it to be otherwise, the original amount of uric acid in the mixed serums must have been about 4 mg. Analysis by the new method and the Folin-Wu method after the addition of oxalate gave values of 3.2 and 4.3 mg. for Serum A, 3.2 and 3.5 mg. for Serum B. Similar treatment of other mixed serums resulted in a yield of separated uric acid which invariably exceeded the Folin-Wu figure. Repeated attempts to obtain larger amounts of uric acid from standard solutions containing 3 mg. in a liter, never resulted in a yield higher than 50 per cent.

It cannot be supposed that the significance of these facts is only a demonstration that the Folin-Wu method gives low results. Such bloods as those of Group 2, Tables I and II, disprove such an idea. There the range of uric acid amount is from 1.6 to 7.9 mg. and the two methods agree. The explanation can only be that

TABLE III.

Identification of Uric Acid Removed as Such from Blood Serum and Uric Acid Solution.

		Uric acid content of solution.	Colorimetric analysis of uric acid separated.	Weight of crystalline precipitate.
		mg.	*mg.*	*mg.*
Serum A.	Folin-Wu	1.4	Twice washed crystals 2.2	
	Folin-Wu following oxalate	4.3	Mother liquor 0.0	3.1
	New method	3.2	Wash water and alcohol 0.02	
Serum B.	Folin-Wu	1.6	Twice washed crystals 2.0	
	Folin-Wu following oxalate	3.5	Mother liquor 0.33	2.2
	New method	3.2	Wash water and alcohol 0.07	
Uric acid standard			Twice washed crystals 1.2	
		3.0	Mother liquor 0.23	2.2
			Wash water and alcohol 0.02	

uric acid is present in the mixed serums and some bloods (Group 1, Tables I and II) in more than one form. In other bloods (Group 2, Tables I and II) there is but one form, or traces only of the second form. In consideration of the fact that the Folin-Wu method and the new method give quite comparable results when

applied to standard uric acid solutions, it must be the second form of uric acid which the Folin-Wu method fails to include while the new method includes it. That it is some form of uric acid rather than any other substance which reacts colorimetrically follows of necessity from the facts here presented that: (*a*) it carries successively through the precipitations with zinc salt and silver magnesium mixture, which are chemically different but equally characteristic; (*b*) it then precipitates quantitatively upon acidification of its solution in the form of crystals which cannot be differentiated from those of uric acid; (*c*) it is changed quantitatively at room temperature in contact with potassium oxalate to a form readily precipitated and extracted by the usual Folin-Wu procedure; and (*d*) the new method gives a value for this second form, as well as the first, in spite of the exclusion of all substances so far tried from the multiplying effect of the cyanide upon the color. Further work to determine the chemical nature of the second form of uric acid is now under way in this laboratory.

As observed above, there is apparently a greater relative amount of the second form of uric acid in serum than in whole blood. We secured freshly drawn samples of blood of sufficiently large volume to allow three sets of analyses by the original Folin-Wu method, Folin-Wu following oxalate method, and the new method. Three determinations were run upon a filtrate from the whole blood, three more upon a filtrate from a serum portion, and another three upon a filtrate from a corpuscle portion. Approximate separation of the bloods was effected by means of the centrifuge. While analysis of the serum portion and corpuscle portion does not furnish strictly quantitative data on the uric acid content of either serum or corpuscles uncontaminated by the presence of small amounts of the other, nevertheless the results unmistakably indicate the order of uric acid distribution between the corpuscles and serum. The figures of Table IV, which represent that distribution in a typical blood specimen would, by more complete separation of the formed elements, be changed so as to further emphasize the fact that the uric acid content of serum is from one and a half to nearly twice that of the corpuscles. Values by the Folin-Wu following oxalate method are 5.7 mg. against 3.0 mg. and the corresponding new method figures are 4.1 mg.

against 2.3 mg. The results obtained when using the original Folin-Wu method are to be considered very approximate since the color due to the small amount of uric acid present was too small for accurate estimation. The second form uric acid, apparent in the table as the difference between the new method and the Folin-Wu method values, is present in the serum portion to an extent three times as great as the first form, 3.2 mg. against 0.9 mg. and in the corpuscle portion is twice as great, 1.5 mg. against 0.8 mg. Such direct observations of the relatively smaller second form uric acid content of corpuscles substantiate the relatively large second form uric acid content of serum previously mentioned. Also we have interpreted these observations, that the added uric acid value is unevenly distributed between corpuscles and serum, as further evidence supporting the existence of the second form of blood uric acid.

TABLE IV.

Distribution of Uric Acid in Blood.

	Folin-Wu method.		Folin-Wu method following $K_2C_2O_4$.		New method.	
Whole blood	2.2		3.9		3.6	
Serum portion	0.9	0.4	5.7	3.0	4.1	2.2
Corpuscle portion	0.8	0.3	3.0	1.4	2.3	1.3

The figures recorded in the first column for each method represent quantities in 100 cc. of serum or corpuscles.

The figures in the second column represent quantities in 100 cc. of blood.

Finally, it should be noted that the distribution of uric acid just described for human blood is in marked contrast with that observed by Benedict[5] for mixed ox blood. In the latter Benedict found all the uric acid (free and combined) in the corpuscles, none in the serum. In the former we find the uric acid in both, but in much greater quantities in the serum.

This different distribution suggests that the second form of uric acid in human blood is probably different from the "combined" uric acid of ox blood. Whether this is the case is only one of the many important questions, which we hope may be attacked by means of the new zinc precipitation-arsenotungstate cyanide

method. We are already studying some of these problems and expect to investigate others as rapidly as new facts concerning the forms of uric acid can be ascertained. Until there is thus developed a more complete understanding of blood uric acid of different species it probably is not desirable to theorize on the physiological and pathological significance of the second form of uric acid in human blood.

Our thanks are due to Dr. H. W. Gauchat of the Cleveland City Hospital for enthusiastic cooperation in securing suitable blood specimens for the particular requirements of this investigation. We gratefully acknowledge the technical assistance rendered by Mr. H. W. Hottenstein. We are also indebted to Dr. E. E. Ecker for making available large quantities of serum from the Wassermann laboratories of Lakeside Hospital and the Cleveland Board of Health.

EXPERIMENTAL RICKETS IN RATS.

III. THE PREVENTION OF RICKETS IN RATS BY EXPOSURE TO SUNLIGHT.*

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Plates 2 and 3.

(Received for publication, October 21, 1921.)

In recent papers it was shown by Hess and Unger that rickets in infants could be cured by frequent short exposures to the sun's rays (1, 2). By this means and without any alteration whatsoever of the dietary, the characteristic signs of this disorder markedly diminish in 3 to 4 weeks, as noted by clinical examination and by the x-ray. As a result of favorable experiences of this nature it was concluded in a study of "the seasonal incidence of rickets" (3) that "hygienic factors, especially sunlight, and not dietetic factors, play the dominant rôle in the marked seasonal variations of this disorder." It seems probable that the ultra-violet rays play a large part in this curative power of the sun, judging from the work of Huldschinsky (4) and others (5, 6, 7) who recently have shown that infantile rickets can be cured by means of the rays produced by the mercury-vapor lamp. In 1918 we tried the curative effect of rays from this source, but, lacking the aid of x-ray examinations, could not convince ourselves of their efficacy; since then we have succeeded in curing rickets by this means.

Having found sunlight efficacious in the rickets of infants, we proceeded to test its value in the prevention of rickets in rats. To this end a series of white rats was placed on the diet (No. 84) described by Sherman and Pappenheimer (8) consisting of patent flour 95.0 per cent, calcium lactate 2.97 per cent, sodium

* Read in abstract before the Society of Experimental Biology and Medicine, October 19, 1921.

chloride 2.0 per cent, and ferric citrate 0.1 per cent. It has been the experience of the investigators in this laboratory that such a diet invariably leads to the development in rats of lesions which are anatomically identical with those of infantile rickets.

In carrying out experiments on rats our practice had been to keep the colony in a semidark room, the yellow shades being drawn at all times. In testing the effect of sunlight, the rats (weighing at the outset about 40 gm.) were kept in absolute darkness, one series being taken out of the room and exposed to the direct sunlight for a period of 15 or 30 minutes. There was no difference whatsoever in the diets of these two groups. After a period of about 3 weeks the animals were radiographed in order to observe early lesions of the epiphysis, and after 30 to 40 days were killed and autopsied. These experiments were begun in April, when the weather permitted four to five exposures a week.

It was found for the first time in our experience that Diet 84, the "rachitic dietary," did not lead to rickets—that the rats which received sun treatment did not show signs of rickets either by x-ray or by histological examination of the bones. It is unnecessary to discuss in detail the histological criteria which we consider characteristic of rickets, as this question has been fully considered in a previous paper (9). It may be stated briefly that they consist of increased width and irregularity of the proliferative cartilage, absence of calcium deposition, and great excess of osteoid in the region of the metaphysis and along the shafts of the bones. It will be seen from Figs. 1 and 3 that the rats which were kept at all times in the dark showed these lesions, whereas the bones of those exposed to the sun did not show them (Figs. 2 and 4).

In the paper previously referred to it was shown that the introduction of 0.4 per cent of secondary potassium phosphate (K_2HPO_4) in place of an equal weight (replacing about one-seventh of the calcium lactate contained in the rickets-producing diet) completely prevented the development of rachitic lesions; this constitutes an addition of 75 mg. of phosphorus per 100 gm. of the diet. In order to test the counterbalancing effect of phosphate and darkness, a series of tests was carried out in the dark with additions of small and increasing amounts of potassium

TABLE I.

Diet.	Duration.	Rat No.	X-ray.	Microscopic examination.
Darkness.				
	days			
No. 84 86 mg. P	34	246	Rickets.	Rickets.
	23	247		"
	22	248		"
72 mg. P		436	Rickets.	
		437	"	
	30	438	"	Rickets.
No. 84 + 25 mg. P	39	262	"	"
	39	263	"	"
	39	264	"	"
	28	443	"	
	28	444	"	Rickets (slight).
	28	445	"	
No. 84 + 75 mg. P	38	121	Negative.	Negative.
	38	122	"	"
	38	123	"	"
Sunlight.				
No. 84 86 mg. P	34	249	Negative.	Negative.
	32	250	"	"
	35	251	"	"
	33	439	"	"
	33	440	"	"
	33	441	"	"
	33	442	"	"
No. 84 + 25 mg. P	39	259	"	"
	39	260	"	"
	39	261	"	"
No. 84 + 75 mg. P	38	124	"	"
	38	125	"	"

phosphate to the standard dietary (No. 84); to one series 25 mg. were added, to another 75 mg. (constituting Dietary 85).

The rats on these diets were kept in the dark but, to serve as control, half of each series was exposed to sunlight for 30 minutes daily when this was possible. As was to be expected in view of our previous experience and the fact that phosphate tends to protect against rickets, none of the rats which were treated with sunlight developed rachitic lesions. Among the group, however, which was kept at all times in the dark, active rickets developed in spite of an addition of 25 mg. of phosphorus. The addition of 75 mg. was found to be sufficient to prevent the development of this disorder. This amount constituted the minimum protective supplement to Diet 84, which in itself contains about 86 mg. of phosphorus. Thus it will be noted that a short exposure to sunlight was equivalent to almost doubling the protective dose of phosphate. If the phosphate content of the diet is adequate, rats do not develop rickets in spite of being kept in the dark throughout the experiment.

The effect of sunlight with other dietaries was also studied, and is being continued. Without entering at this time into a detailed discussion of their influence, it may be of interest to record the observation that in one series of animals where 10 per cent of egg albumin was substituted for an equivalent amount of flour, rickets developed in some of the rats in spite of the sunlight treatment; whether this is to be attributed to a reduction of phosphate incidental to diminishing the percentage of flour, or to the injurious effect of the egg albumin itself, will be determined by experiments which are in progress. Possibly the increased rate of growth of these animals with the accompanying increased phosphorus requirement may have been a factor of moment.

DISCUSSION.

As sunlight has a marked effect on the bony development of rats, it is evident that in future in similar nutritional investigations, the light factor will have to be controlled and standardized. It seems probable that some of the irregularities and lack of conformity observed by investigators in this field may be attributed to keeping the experimental animals under dissimilar intensities

of light. The most interesting aspect of the question, however, is the phenomenon that the sun's rays are able to stimulate a deposition of inorganic salts where these are lacking. The damaging effect of darkness emphasizes the fact that sunlight is of great importance, not merely for the vegetable world but also for the higher animals. Furthermore, the fact that sunlight is efficacious in the rickets of both human beings and rats, serves to show the similarity of this disorder in these two species. These results indicate that in the prevention and causation of rickets at least one hygienic factor plays an important rôle which will have to be carefully considered in future studies of this disorder.

CONCLUSIONS.

Rachitic lesions which develop regularly in rats upon a diet adequate in calcium but low in phosphorus, may be prevented by short exposures to direct sunlight.

This protection is equivalent to the addition of at least 75 mg. of phosphorus to the diet in the form of basic potassium phosphate.

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EXPLANATION OF PLATES.

Plate 2.

Fig. 1. Rat 246. 34 days on Diet 84. Darkness. Chondrocostal junction showing advanced rickets. (Silver nitrate—Van Gieson stain.)

Fig. 2. Rat 249. Same litter as Rat 246. 34 days on Diet 84. Sunlight. Chondrocostal junction showing no rickets. (Silver nitrate—Van Gieson stain.)

Plate 3.

Fig. 3. Rat 263. 39 days on Diet 84 plus 25 mg. of P added as K_2HPO_4. Darkness. Radiograph showing rachitic changes at knee-joint.

Fig. 4. Rat 261. Same litter as Rat 263. 39 days on identical diet. Sunlight. No rickets.

FIG. 1.

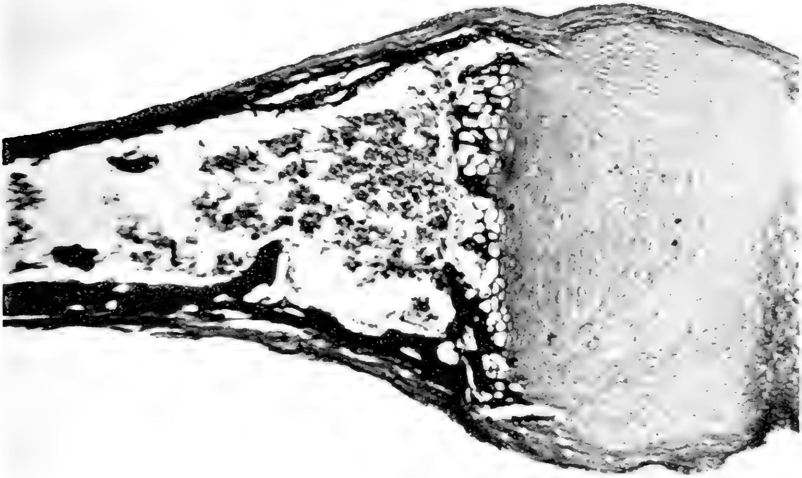

FIG. 2.

(Hess, Unger, and Pappenheimer: Experimental rickets in rats. III.)

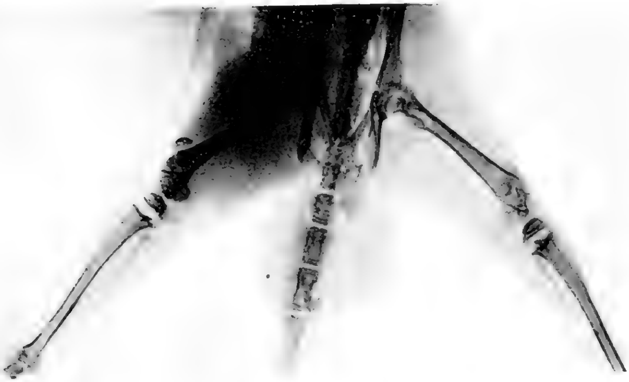

FIG. 3.

FIG. 4.

(Hess, Unger, and Pappenheimer: Experimental rickets in rats. III.)

SOME HUMAN DIGESTION EXPERIMENTS WITH RAW WHITE OF EGG.

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(Received for publication, November 16, 1921.)

The behavior of raw white of egg in the alimentary tract of the dog has been studied extensively by Bateman[1] but there does not appear to be in the literature any corresponding investigation in regard to the digestibility of white of egg in this state by the human subject. Falta[2] reports two experiments in which dried egg albumin (presumably raw) was added to a basal ration, and the amount of nitrogen in the feces determined.

TABLE I.

Experiment No.	N intake on basal ration.	With egg added.	Per cent of N from egg albumin.	Total N in feces.		Per cent of intake lost in feces.	
				On basal ration.	With egg added.	On basal ration.	With egg added.
	gm.	*gm.*		*gm.*	*gm.*		
1	16.40	27.68	40.0	1.12	1.81	7.0	6.5
2	19.20	36.12	47.0	6.05	6.25	31.0	17.3

From these figures it appears that the addition of considerable amounts of dried egg albumin (80 gm. in the first experiment and 120 gm. in the second) instead of depressing the coefficient of digestibility actually raised it when the larger amount of egg was taken, the coefficient on the basal ration in the second experiment being 69 per cent and on the egg ration 83 per cent.

Falta followed the nitrogen elimination in the urine and found that the highest point was reached somewhat later for egg albumin

[1] Bateman, W. G., *J. Biol. Chem.*, 1916, xxvi, 263.

[2] Falta, W., *Deutsch. Arch. klin. Med.*, 1906, lxviii, 517.

than for gelatin or casein. It seems clear that native egg white offers some resistance to the speedy action of the digestive enzymes, but as Bayliss[3] has shown, though trypsin acts more quickly at first on cooked egg albumin, it will if sufficient time be allowed digest the uncooked as completely as the cooked.

Wolf and Österberg[4] studying primarily the urinary nitrogen and sulfur on diets in which various protein foods were in turn added to a simple mixed ration, determined nitrogen in food and feces and found in one case with a total intake of 23 gm. of nitrogen 70 per cent of which was derived from raw egg white, the loss of nitrogen in the feces was 41 per cent of the intake; but in another period, with total intake of 14 gm. and 51 per cent of the nitrogen from the egg white, the loss was only 15 per cent of the total intake or about the same as on the basal ration alone. It would scarcely seem fair to draw conclusions from these two conflicting experiments.

The authors have accordingly conducted experiments on ten subjects,[5] all healthy young women, who took daily from ten to twelve whites of eggs as a part of a simple mixed diet, first cooked, in a 3 day period, then raw for the same length of time. The diet was uniform throughout the experiment and furnished 67 gm. of protein, to which the egg whites contributed 48 gm. or 70 per cent of the total. The experiments were divided into three groups, one in which the raw egg whites were taken thoroughly beaten, one in which they were taken in their natural state, and a third in which half were beaten and half unbeaten. In no case was there any sign of indigestion, such as discomfort or diarrhea, though one or two subjects found them slightly laxative. The cooked eggs were never subjected to a temperature or method of cooking (such as frying) which would render them tough or otherwise interfere with ease of digestion.

Coefficients of digestibility have been calculated for the total protein of the diet, which seems the fairest way to judge experiments of this sort; and also in the conventional way for the egg

[3] Bayliss, W. M., The nature of enzyme action, London, 1908, 148.

[4] Wolf, C. G. L., and Österberg, E., *Biochem. Z.*, 1912, xl, 234.

[5] Some of these experiments have been reported in a preliminary paper (Rose, M. S., and MacLeod, G., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 119).

protein alone, making average allowance for the loss of the protein of the other foods in the diet.

The cooked eggs were uniformly well digested, coefficients ranging from 83 to 91 per cent with an average of 86 per cent for the diet as a whole; or from 82 to 93 per cent with an average of 86 per cent for the egg white alone.

On the whole the raw whites were well utilized, the average difference between the cooked and raw being only 4 per cent for the protein of the whole ration or 5.5 per cent for the egg white protein alone, in favor of the cooked.

The differences between the cooked and the raw whites varied with the mode of preparation, those beaten light being the best utilized, and those taken in the natural state least well absorbed, as shown by Table II.

TABLE II.

Difference between Coefficients of Digestibility of Egg Whites in Favor of Cooked Whites.

Group No.	Mode of preparation.	Per cent of difference.	
		For whole ration.	For egg protein alone.
I	Unbeaten.	+6.8	+9.6
II	Half beaten and half unbeaten.	+3.3	+4.6
III	Beaten light.	+1.7	+3.0

EXPERIMENTAL.

The daily ration consisted of the same foods in all ten cases, but the proportions of the individual foods differed slightly, as indicated below.

Analyses for total nitrogen were made in the laboratory and protein calculated as N $\times$ 6.25.

The feces were marked off by carmine, and analyzed for total nitrogen in 3 day periods. The output of each subject is given in Table IV.

The coefficients of digestibility calculated from the foregoing are given in Table VI. In estimating the coefficients for the egg white alone, the following arbitrary allowances have been made for loss in digestion of the protein of the other items in the diet.

TABLE III.

Daily Intake of Food.

Food materials.	For Subjects G. S., M. K., L. S., M. F., M. R., F. R.		For Subjects G. B., E. B., M. E., D. T.	
	Weight of food.	Protein.	Weight of food.	Protein.
	gm.	*gm.*	*gm.*	*gm.*
Egg whites	372	47.66	375	47.63
Rice	85	6.80	85	8.75
Cream	123	2.72	79	1.98
Saltines	28	3.05	35	4.68
Lettuce	85	1.49	85	1.00
Fruit juice	600	5.06	600	3.07
Butter	39	0.39	39	0.39
Olive oil	33		33	
Sugar	50		50	
Total		67.17		67.47

TABLE IV.

Daily Output of Nitrogen in Feces Calculated to Protein.

Group No.	Subject.	Nitrogen in feces × 6.25.	
		Raw egg diet.	Cooked egg diet.
		gm.	*gm.*
I (Raw whites, unbeaten).	M. F.	11.24	16.35
	E. B.	10.00	8.13
	M. E.	15.25	8.43
II (Raw whites, half beaten, half unbeaten).	G. S.	9.59	11.70
	L. S.	9.29	11.78
	M. R.	8.19	11.56
	F. R.	11.26	12.22
III (Raw whites, beaten light).	M. K.	11.31	10.83
	G. B.	12.56	10.26
	D. T.	7.94	6.30

TABLE V.

Food material.	Allowances for loss in digestion of protein.
	per cent
Rice	17
Saltines	12
Lettuce	17
Cream	3
Butter	3
Fruit juices	15

TABLE VI.

Coefficients of Digestibility for Raw and Cooked Egg Whites.

Group.	A. For total protein of ration.				B. For protein of egg whites only.		
	Subject.	Cooked egg white.	Raw egg white.	Difference in favor of cooked egg white.	Cooked egg white.	Raw egg white.	Difference in favor of cooked egg white.
		per cent	per cent	per cent	per cent	per cent	per cent
I (Raw whites, unbeaten).	M. F.	83.3	75.7	+ 7.6	81.9	71.2	+10.7
	E. B.	87.9	85.3	+ 2.7	88.7	84.8	+ 3.9
	M. E.	87.5	77.4	+10.1	88.1	73.8	+14.3
Average for Group I.		86.2	79.4	+ 6.8	86.2	76.6	+ 9.6
II (Raw whites, partly beaten and partly unbeaten).	G. S.	85.7	82.6	+ 3.1	85.4	81.0	+ 4.4
	L. S.	86.2	82.5	+ 3.7	86.0	80.8	+ 5.2
	M. R.	87.9	82.9	+ 5.0	88.8	81.2	+ 7.0
	F. R.	83.3	81.9	+ 1.4	81.8	79.8	+ 2.0
Average for Group II.		85.8	82.5	+ 3.3	85.3	80.7	+ 4.6
III (Raw whites, beaten light).	M. K.	83.2	83.9	− 0.7	81.8	71.2	− 1.0
	G. B.	84.8	81.4	+ 3.4	84.2	79.4	+ 4.8
	D. T.	90.6	88.2	+ 2.4	92.5	89.1	+ 3.4
Average for Group III.		86.2	84.5	+ 1.7	86.8	83.8	+ 3.0
Average for all cases.		86.0	82.0	+ 4.0	85.9	80.4	+ 5.5

CONCLUSIONS.

Raw whites of eggs, in as large amounts as ten to twelve whites daily, are well utilized in the human subject, the average coefficient of digestibility calculated for the raw egg white alone being 80 per cent as compared with 86 per cent for cooked whites in the same diet. The absorption varies with the method of preparation, being less for raw egg whites taken in their natural state than when beaten light. A mixture of whites partly beaten and partly unbeaten gave an intermediate value. The quantities consumed are regarded as maximal in dietary practice, and it seems unnecessary to emphasize the difference between raw and cooked eggs if the raw eggs are beaten.

A MODIFICATION OF FOLIN'S COLORIMETRIC METHOD FOR THE DETERMINATION OF URIC ACID.*

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INTRODUCTION.

In 1912, Folin and Denis (1) introduced phosphotungstic acid as a color reagent for the detection of uric acid, and from Folin's laboratory, within a year, appeared methods for the quantitative estimation of uric acid in urine (2, 3) and blood (4). The method was greatly improved by Benedict and Hitchcock's (5) discovery that cyanide very considerably increased the intensity of the color developed and retarded the fading, and recently, the method[1] has been still further improved by Folin and Wu (6). Volumetric methods have been proposed by Curtman and Lehrman (7) and Morris (8), but the colorimetric procedure has been the method most commonly used.

* A preliminary report of this work appears in *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 126.

[1] Transfer a measured volume of blood into a flask having a capacity of fifteen to twenty times that of the volume taken. Dilute the blood with 7 volumes of water and mix. With an appropriate pipette, add 1 volume of 10 per cent solution of sodium tungstate ($Na_2WO_42H_2O$), and mix.

With another pipette, add to the contents of the flask (with shaking), 1 volume of $\frac{2}{3}$ normal sulfuric acid. Close the mouth of the flask with a rubber stopper and give a few vigorous shakes. Much oxalate or citrate interferes with the coagulation and later with the uric acid determination. 20 mg. of potassium oxalate are ample for 10 cc. of blood. When the blood is properly coagulated, the color of the coagulum changes from pink to dark brown. If this change does not occur, the coagulation is incomplete, but the sample may be saved by adding 2 N sulfuric acid, drop by drop, shaking vigorously after each addition, and allowing the mixture to stand for a few minutes before adding more, until the coagulation is complete. Pour the mixture on a filter paper large enough to hold the entire contents of the flask and cover with a watch-glass. The filtrate should be water-clear.

The method is simple and accurate and is admirably adapted to the tungstic acid protein-free filtrate so widely used in blood analysis.

There are, however, two disadvantages which in routine practice are of some importance; first, the relatively slight intensity of the color developed with such amounts of uric acid as are in normal blood; and secondly, the troublesome crystalline precipitate which often appears in the colored solution, thereby rendering reading impossible without filtration, a procedure which somewhat diminishes the color. We have endeavored to overcome these difficulties and believe we have succeeded.

In an effort to find the cause for the troublesome precipitate developing in the colored solution, we found that if Folin's uric acid reagent were dialyzed in heavy parchment membranes against large amounts of tap water, until all the free acid was gone, and the solution so dialyzed was evaporated to dryness, a reagent was obtained which, in the presence of uric acid and an excess of NaCN, gave a very intense color and a more or less dense flocculent precipitate. The latter did not alter in amount or character over a period of 24 hours or more. If, on the other hand, Folin's uric acid reagent were boiled cautiously to dryness without dialysis, a reagent was obtained, which, in the presence of uric acid and an excess of sodium cyanide gave the same intense color, but also a dense crystalline precipitate in the course of 3 to 5 minutes. This last reagent, which we call sodium phosphotungstate "B" when mixed with the dialyzed sodium phosphotungstate "D" will cause dissolution of the flocculent precipitate and at the same time, no crystalline precipitate will develop unless too much "B" is added. The proper mixture of these two-salts results in a reagent which under conditions of the determination, gives a color nearly five times as intense as that given by Folin's procedure and no precipitate results.

Preparation of Phosphotungstic Reagents.

Preparation of Phosphotungstate "B".—1,000 cc. of Folin's reagent[2] in a large porcelain casserole are boiled rapidly over a

[2] Sodium tungstate (100 gm.), 85 per cent phosphoric acid (80 cc.), plus water (700 cc.) boiled with reflux condenser for 24 hours. Cooled and diluted to 1 liter.

free flame until the volume is about 400 cc. The size of the flame is then reduced and boiling gently takes place, until the whole is the consistency of pea soup. If the solution turns green, decolorize with bromine water. At no time should the temperature of the fluid rise above 110°C. Cool in the ice box or ice solution until the solution is about 10°C. A mass of heavy yellow crystals will separate out. Allow them to settle. Pour the supernatant syrupy liquid through a Buchner suction funnel and filter the crystals off in the same funnel. Suck as dry as possible with tamping and strong suction, continuing suction for 3 to 4 hours. Then dry on filter paper over night in an incubator (37°C.). The crystals are sodium phosphotungstate "B", contaminated with a small amount of sodium phosphate. They should be perfectly dry. Yield 90 gm.

Preparation of Phosphotungstate "D".—1,000 cc. of Folin's solution are placed in a sac of "special" parchment paper, capable of holding 4 liters, and dialyzed against 10 liters of tap water for 5 days. The water should be changed once a day. Otherwise, the procedure needs no attention. The solution in the sac will increase to about 3,000 cc. At the end of 4 or 5 days, titrate 5 cc. of the solution with 0.1 N NaOH, using phenolphthalein as the indicator. 5 cc. of a 20 per cent solution of "D" neutralizes about 15 cc. of 0.10 N NaOH. To be sure that all the free phosphoric acid has gone from the dialyzed fluid, it is necessary to titrate. The total titration value of the solution should be 1,000 to 1,600 cc. If more alkali is needed, dialyze another day to get rid of the remaining phosphoric acid. If not, proceed as below.

Transfer the solution in the sac to a large casserole and evaporate over a free flame. If the solution turns green, decolorize with bromine water as often as necessary. As the amount of solution approaches 400 cc. the flame is lowered, and the boiling takes place cautiously, until the amount of solution is about 200 cc. The solution is now transferred to the steam bath, and evaporation continued until solid material begins to separate out. Do not allow the solid mass to become hard, so that it cannot be broken easily with a stirring rod. When *almost* dry, that is, when no free liquid is seen, break up into small lumps and complete the drying in the air with occasional breaking up of the

lumps and occasional heating in the steam bath. If dried *completely* without breaking up, the mass becomes stony hard and is very difficult to get out of the casserole. This salt is sodium phosphotungstate "D". Its properties will depend on the dialyzing paper used in its preparation. The heaviest grade of dialyzing paper made by the Reeve-Angel Company, 7 Spruce Street, New York City, is satisfactory. In this laboratory we have used a very heavy Belgian parchment paper, purchased before the war. It has given far better results than any other paper, but we have been unable to duplicate it. Celloidin sacs are useless, as the reagent passes rapidly through such membranes. Ordinary dialyzing papers yield only "B", or "B" and "D" mixed. Papers otherwise unsatisfactory had been made better by coating the inner surface of the paper with a thick celloidin membrane, but we have not employed this procedure sufficiently to speak with assurance as to its value. The longer the dialysis the more intense are the specific properties of "D", as distinguished from those of "B", but also the smaller the yield. 5 days dialysis is the average time necessary for 1,000 cc. of Folin's solution. Smaller quantities take a shorter time and *vice versa.* In case of doubt, it is better to dialyze another day. If any free phosphoric acid is left in the solution, the reagent is spoiled in evaporation. Yield about 85 gm.

Preparation of the Reagent Mixture of "B" and "D".

Make a 20 per cent solution of "B" water. Also make a 20 per cent watery solution of "D". Decolorize with bromine water, if not clear yellow. Boil off the excess of bromine. "B" will dissolve in water completely giving a perfectly clear solution. There is usually an insoluble residue in "D", and the solution should be warmed and filtered. If the "B" salt alone be used in analysis, a crystalline precipitate will form in the final colored solution on standing. Should the "D" salt alone be used, no *crystalline* precipitate will form, but a more or less dense *flocculent* precipitate will form immediately. When the "D" salt is pure this precipitate will remain unaltered for 24 hours or more. This flocculent precipitate undergoes dissolution giving a clear solution if some of the "*B*" be added. If too *little* "B" be added, the flocculent precipitate will not disappear; if too *much,* it will

disappear rapidly and the crystalline precipitate will subsequently form. The crystalline precipitate will form early, in case much "B" has been added, later, if less. There is a point, however, where a solution can be made that will give a clear solution in which the crystalline precipitate will not develop for at least 48 hours, and there is a considerable range on either side of this point where solutions quite satisfactory for ordinary use can be made, in which the precipitate will not develop for more than 12 hours under the conditions of the analysis. As a rule, from $\frac{1}{4}$ to 1 part of "B" to 1 part of "D" is satisfactory. Mix the "B" 20 per cent solution in definite proportions, as follows:

5 cc. "B" + 20 cc. "D" = 1:4.
5 " "B" + 15 " "D" = 1:3.
5 " "B" + 10 " "D" = 1:2.
5 " "B" + 5 " "D" = 1:1.

In each of four small Erlenmeyer flasks put

$\frac{1}{4}$ cc. Benedict's standard, measured roughly.
3 " 5 per cent sodium cyanide.
5 " distilled water.
2 " 10 per cent NaCl in 0.1 N HCl.

To each flask add 1 cc. of the above mixture of "B" and "D". A flocculent precipitate should develop in each flask. Let the flasks stand and watch carefully for the development of a crystalline precipitate. Choose for the final solution that proportion of "B" and "D" which remains *clear* for $\frac{1}{2}$ hour or *more*. If all of them precipitate inside that time, the "D" salt has been improperly prepared. If the solutions are not clear of the flocculent precipitate a still *greater* proportion of "B" should be used. Should all the test flasks precipitate, the dialyzed reagent may, of course, be used without any addition of "B". 5 cc. of this "special reagent" are ample to produce the full color with 1 mg. of uric acid under the conditions of the determination.

Use of Reagents for Determination of Uric Acid in Blood.

The essential points of difference between our modification and the original method are as follows:

1. No sodium carbonate is used, sodium cyanide furnishing the requisite alkalinity.

2. The solution in which the color is to be developed must be diluted to a definite volume.

3. The solutions must be diluted at accurate intervals after the reagent is added.

4. A specially prepared uric acid reagent must be used. A protein-free filtrate is obtained with tungstic acid according to the method proposed by Folin and Wu (6).

For normal bloods, 20 cc. of filtrate, equivalent to 2 cc. of blood, are used, and for bloods suspected of having over 10 mg. of uric acid per 100 cc., 10 cc. or even 5 cc. may be used, if economy of blood be desirable.

To the protein-free filtrate in a 35 cc. centrifuge tube, add 5 cc. of 5 per cent silver lactate in 5 per cent lactic acid. Stir thoroughly with a fine glass rod. Wash the rod into the tube with a few cc. of water, and centrifuge 2 to 3 minutes. The supernatant liquid should be clear. Add a few drops of silver lactate. If a precipitate forms, add 2 cc. more of silver lactate, stir and centrifuge again. Decant the supernatant liquid and drain as completely as possible. Add 2 cc. of 10 per cent NaCl in 0.1 N HCl to the precipitate. Let the solution run into the middle of the precipitate, and not down the side of the tube, a procedure which tends to make the precipitate creep. Break up the precipitate very thoroughly with a fine glass rod.

So far the procedure is that of Folin and Wu. Now add 4.5 cc. of water as accurately as possible with a Folin pipette, and stir again very thoroughly. Wash the rod with 0.5 cc. of water from the Folin pipette. Centrifuge rapidly for 5 minutes. Now transfer the supernatant liquid quantitatively to a 25 cc. flask. This is best accomplished by holding the flask and a fine glass rod, whose tip just touches the side of the flask neck, in the left hand and pouring with the right. When the bulk of the liquid has drained out, touch and retouch the lip of the tube to the rod, until no liquid adheres to the rod as the tube is taken away on several trials. With the rod still in position, run in (down the rod to wash it) 3 cc. of 5 per cent NaCN. This should be accurate to 0.1 cc. Drain the rod and take it out.

Now prepare two standards in 50 cc. flasks. To one flask, add 0.5 cc. of Benedict's standard,[3] to another 1.0 cc. Now add 9.5 and 9.0 cc. of water, respectively, and to each flask 4 cc. of 10 per cent NaCl in 0.1 N HCl, and exactly 6.0 cc. of 5 per cent NaCN.

To the unknown, add 1.5 cc., and to the standards 3 cc. of the special reagent described below. Rotate each flask briskly to insure complete mixture. The additions of the reagent should be made as nearly simultaneously as possible. It is perhaps better to add the reagent at minute intervals and dilute at corresponding times. Let the flasks stand 10 minutes by the clock and dilute to the mark in the same order as the reagent was added. The colorimetric estimations may be made immediately after dilution.

The calculation is made according to the formula:

$$\frac{R \times a \times b}{R_1 \times 2},$$

where R is the reading of the standard, a the figure by which it is necessary to multiply the sample taken to make 100 cc. of blood (50 in case 20 cc. of filtrate are taken), b the amount of uric acid in the standard, expressed in milligrams, and R_1 the reading of the unknown. The equation must be divided by 2, since the unknown is in a 25 cc. flask, while the standard is in a 50 cc. flask.

The color develops slowly, the maximum not being reached in 10 minutes, but the color is sharply proportional to the amount of uric acid present. The depth of color is dependent on the *concentration* of the cyanide in the final solution. Cyanide alone in this concentration gives no color until 8 to 12 hours later, when a

[3] Benedict's standard uric acid solution is prepared as follows: 9 gm. of pure crystallized disodium hydrogen phosphate, together with 1 gm. of crystallized sodium dihydrogen phosphate, are dissolved in 200 cc. of hot water and the solution is filtered, if not perfectly clear. The filtrate is made up to a total volume of about 500 cc. with hot water, and this hot solution is poured upon exactly 200 mg. of pure uric acid, suspended in a few cc. of water in a liter volumetric flask. The mixture is agitated for a moment or two, until the uric acid completely dissolves, and then cooled. Exactly 1.4 cc. of glacial acetic acid are added, and the flask is diluted to the mark and mixed. 5 cc. of chloroform are then added to prevent the growth of bacteria or molds. 5 cc. of this solution contain exactly 1 mg. of uric acid. This solution keeps perfectly well for at least a month.

faint tinge of blue develops. A much greater concentration of cyanide will develop a blue color without uric acid. The color continues to increase very gradually over a period of many hours. The increase is proportional in the standard and the unknown. No carbonate is used. Folin's uric acid standard cannot be used since the presence of sulfite prevents the development of the deep color If *serum*, rather than whole blood, be used, the determination may be made after the plan for urine (see below). We have been unable thus far to find polyphenols in *serum*. That portion of the precipitate from silver lactate which remains behind after liberation of the uric acid with HCl has never given any color with the reagent when serum was used. Apparently, the polyphenols are confined to the cells.

Use of Reagents for the Determination of Uric Acid in Urine.

The same principles of modification apply to the analysis of urine as to the analysis of blood. Very careful control of dilution and accurate time intervals are required and again cyanide is the only alkali used.

Add to 1 or 2 cc. of urine according to concentration in a 35 cc. centrifuge tube 6 cc. of water. Then add 5 cc. of silver lactate, stir and centrifuge. Decant the supernatant liquid. To the precipitate add exactly 4 cc. of 5 per cent NaCN and exactly 10 cc. of water. Stir until all the precipitate is dissolved.

Prepare in two 100 cc. flasks suitable standards. To one flask add 2 cc. of standard, to another 4 cc. of standard and 8 and 6 cc. of water, respectively. To each standard add 4 cc. of cyanide. Then with time intervals as with blood, add 5 cc. of special reagent to each flask, and to the unknown in the tube. Mix thoroughly. Allow each to stand exactly 10 minutes, and dilute to the mark. The unknown can be poured into the 100 cc. flask just before the 10 minutes are up and the washings serve to dilute it at the right time.

Using 2 cc. of urine in a 100 cc. flask, and 2 and 4 cc. of standard in a 100 cc. flask, a range of from 0 to 60 mg. of uric acid per 100 cc. is covered. With 1 cc. of urine and the same standards a range of from 60 to 120 mg. per 100 cc. is covered.

If there is albumin in the urine, it must be removed by heat and acetic acid, or tungstic acid, as even very small amounts of albumin prevent the proper precipitation of uric acid by silver lactate.

EXPERIMENTAL.

When phosphotungstate "B" is used alone as the reagent for uric acid a crystalline precipitate develops very quickly; a flocculent precipitate develops when phosphotungstate "D" is used alone. On the other hand, the effect on the formation of a precipitate of combination of phosphotungstate "B" and "D" is quite striking for it has been possible to find a proportion between the two salts which keeps the solution clear (Table I). The proportion of the two salts which gives the best results may vary with the different lots.

The depth of color is dependent upon the concentration of the cyanide radical. The tendency to precipitate is determined by

TABLE I.

Effect of Varying Amounts of "B" and "D' on Time of Appearance of Precipitate.

Proportion "B" to "D."	Precipitate appeared.
1:0	3½ minutes.
4:1	16 "
2:1	22 "
4:3	28 "
1:1	60 "
1:3	No precipitate.
0:1	Always cloudy. No crystalline precipitate.

the OH ion concentration. From these facts it might be inferred that Folin's original solution could be used with NaCN as the only alkali. But while increased color and freedom from precipitate can be obtained in this way, such a large experimental error is introduced that the method is quite worthless, except when the readings of the standard and the unknown are very close together. This is shown in Table II. Method 1 indicates the method described in this paper. Method 2 indicates the use of Folin's reagent with NaCN as the only alkali. Method 3 indicates the regular method of Folin and Wu.

In the above experiments, the same blood filtrate was used throughout and varying amounts were taken for analysis in order to vary the readings of the unknown in comparison with that of the standard. It will be seen that unless the readings are close

together very large errors are introduced when Folin's solution is used with cyanide alone.

TABLE II.

Comparison of Methods Using NaCN as the Only Alkali.

Method.	$\frac{\text{Reading of standard}}{\text{Reading of unknown}}$.	Amount of uric acid.
		mg. per 100 cc.
1	$\frac{20}{22}$	3.0
2	$\frac{20}{22}$	3.0
3	$\frac{20}{22}$	3.0
1	$\frac{20}{33.5}$	2.9
2	$\frac{20}{44.0}$	2.2
1	$\frac{20}{13.4}$	3.0
2	$\frac{20}{9.5}$	4.2

TABLE III.

Rate at Which the Color Develops.

Solution made.	Diluted.	Interval.	Reading.
a.m.	*a.m.*	*min.*	
10.45	10.50	5	20.0
10.47	10.53	6	20.0
10.49	10.56	7	20.0
10.50	11.00	10	20.0
11.45	11.50	5	20.2

Development of the Color.

The color develops slowly and flasks should stand before dilution for 10 minutes. The rate of increase of color has by this

time fallen off so much that a considerable error in time elapsed before dilution does not introduce an appreciable variation in the reading, as may be seen in Table III.

Standards and unknowns made up in 1 hour are comparable with one another.

Solution made.	Reading.
12.15	20.0
12.45	20.0
1.00	19.9
2.15	21.6

The intensity of the color is, as we have said, dependent on the concentration of the cyanide radical. A considerable variation in cyanide concentration, however, is consistent with accurate readings. Flasks containing 1 cc. of standard, 5 cc. of water, and 1.5 cc. of reagent were made alkaline with varying amounts of cyanide. See Table IV.

TABLE IV.

Effect of Varying Amounts of Cyanide.

Cyanide.	Reading.
cc.	
3.0	20.0
3.1	19.9
3.2	20.0
3.5	20.0
4.5	18.9

From the foregoing table, it is evident that when the method calls for 3 cc. of NaCN, a variation of ± 0.5 cc. does not alter the result. However, in our work we measure the cyanide solution to within ± 0.1 cc.

Sodium carbonate or other strong alkali prevents the development of the deep color, and sodium sulfite lessens the color even more, so that Folin's sulfite standard cannot be used in our method. The color develops slowly and does not reach a maximum for an hour or more, but there is a sharp proportionality between the amount of uric acid and the depth of color.

Accuracy.

We have taken as our standard of accuracy, Folin's method. The method we propose, we believe is quite as accurate as Folin's. The analyses in Table V, taken from a large series of determinations, serve as examples.

TABLE V.

Folin's Method Compared with That of the Authors, Milligrams of Uric Acid in 100 Cc. of Blood.

Folin's method.	Authors' method.
23.3	23.2
15.0	15.0
15.2	15.0
3.0	3.0
3.4	3.3

Specificity.

The reagent, prepared as we have suggested, reacts towards organic reducing substances in alkaline solution exactly as does Folin's solution.

Composition of Phosphotungstates.

We have been unable to make satisfactory analyses of the two salts used in preparing the reagent. Our attempts in this direction have convinced us that neither is a pure chemical substance. Both give indications of being mixtures of two or more compounds.

Sodium phosphotungstate "B" can be easily recrystallized from water or alcohol. Sodium phosphotungstate "D" is very difficult to purify and seems to crystallize fractionally as if it were composed of several compounds. We have prepared the free acid from sodium phosphotungstate "B". Furthermore, we have prepared in a comparatively pure state, the blue reduced tungstate. It seems to be stable in air and is blue-black in color. This was prepared by evaporating a solution of the salt in the presence of metallic zinc in an evacuated desiccator containing a strong solution of pyrogallic acid in strong NaOH.

SUMMARY.

A modification of Folin's method for the determination of uric acid in blood and urine is described. In our hands, this modification eliminates the two disadvantages, the faint color, and the precipitate in the final solution for colorimetric estimation, of the original procedure.

The color developed by our method is nearly five times as great as that developed on Folin's method, and does not fade over a period of several hours. In fact the color increases gradually and proportionally in both standard and unknown, so that when standard and unknown are made up at the same time, they may be read at any time during the next 2 hours or more. No crystalline precipitate develops in the colored solution.

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AMINO-ACIDS IN NUTRITION.

IV. A MODIFIED BIOLOGICAL METHOD OF STUDYING AMINO-ACID DEFICIENCIES IN PROTEINS. CYSTINE AS A GROWTH-LIMITING FACTOR IN THE PROTEINS OF THE GEORGIA VELVET BEAN (STIZOLOBIUM DEERINGIANUM).

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(Received for publication, October 19, 1921.)

Employing essentially the technique of McCollum and Davis (1), using the seed as the only source of protein, and satisfying all the other dietary essentials in the rations, the author attempted to make amino-acid additions to the pea (*Vicia sativa*) as a part of his general scheme of studying the capacity of the animal organism to synthesize the pyrrolidine nucleus of the protein molecule (2), but in that study has met with no positive results. This paper will show, by a modified method of procedure, the practicability of making amino-acid additions to such complex substances as seeds.

Waterman and Jones (3), in a recent communication, state that amino-acid deficiencies in the Chinese and Georgia velvet beans cannot account for the failure to promote growth; for analyses (4, 5) have shown the above mentioned proteins to be adequate except possibly with respect to cystine; and no improvement resulted from the addition of this amino-acid.

It will be shown in the following pages by the modified procedure adopted by the author that *cystine is unquestionably a growth-limiting factor in the proteins of the Georgia velvet bean*, which could not have been found by employing previous methods of making straight amino-acid additions. The results of the experiments are given in the following charts.

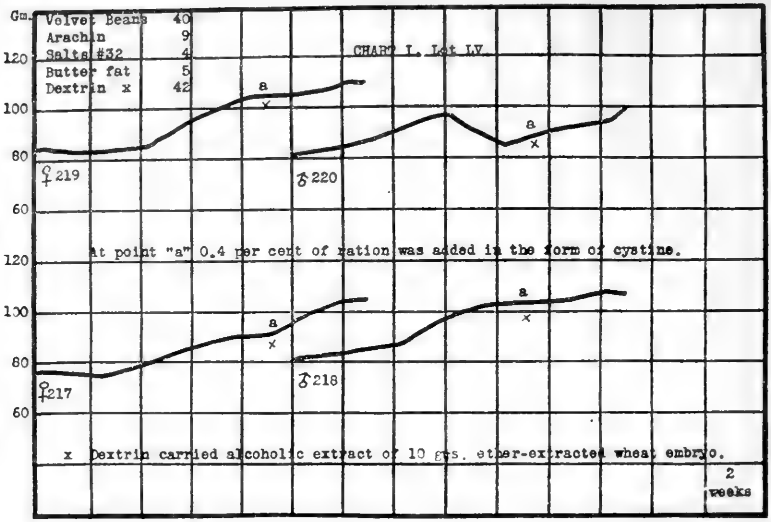

CHART I, LOT LV. This chart indicates that when Georgia velvet beans are fed as the only source of protein at a level of 40 per cent, very little growth takes place, and that arachin, one of the globulins and the main protein from the peanut, does not furnish amino-acids to supplement those deficient in the velvet beans. At point "a" 0.4 per cent of the ration was added in the form of cystine, but no response was obtained.

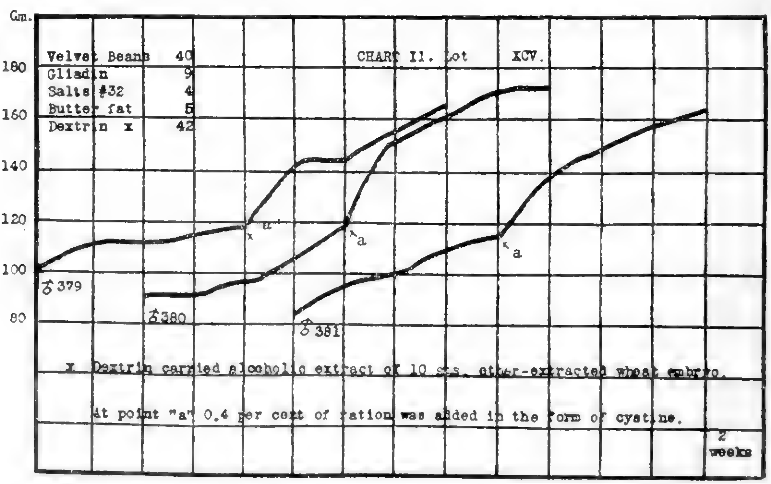

CHART II, LOT XCV. This experiment demonstrates that, in the presence of gliadin, there is a definite response to cystine addition to the velvet bean proteins, which is not, however, very marked 2 weeks after this amino-acid addition.

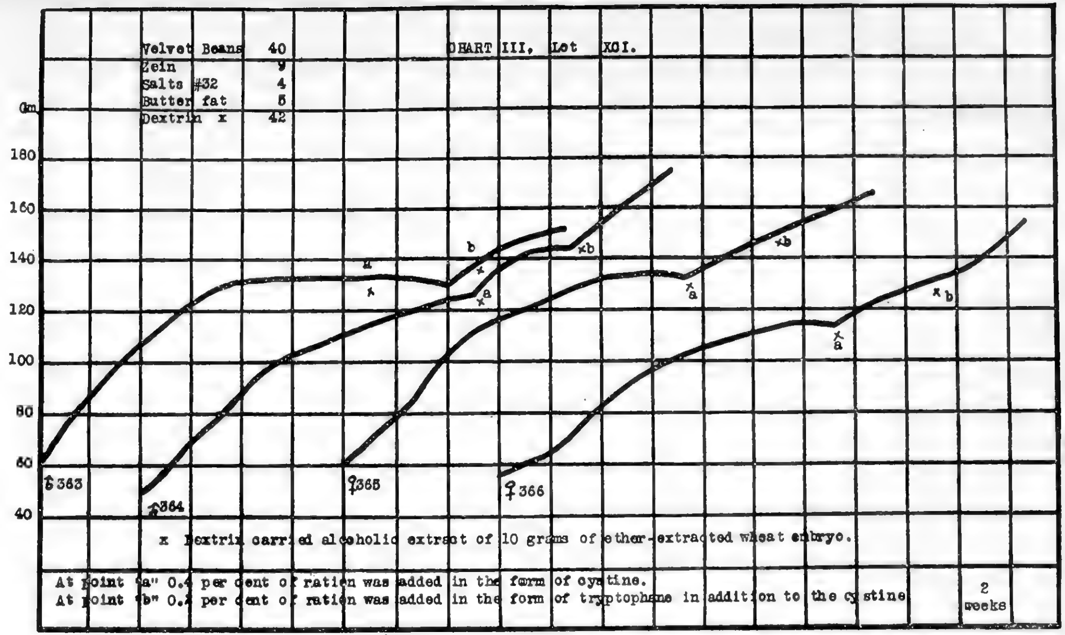

Chart III, Lot XCI. Zein seems to supplement the proteins of the Georgia velvet bean in the earlier periods of growth, but, it will be noted that after 8 weeks the nature of growth is considerably retarded. The addition of cystine at point "a" brought about a slow but definite improvement in growth with no further increase in the character of growth on the addition of tryptophane to the cystine at point "b". This experiment and the preceding, Lot XCV, strongly suggest that cystine is a growth-limiting factor in the proteins of the Georgia velvet bean, which becomes apparent only after other amino-acids are satisfied as supplied by such deficient proteins as gliadin and zein.

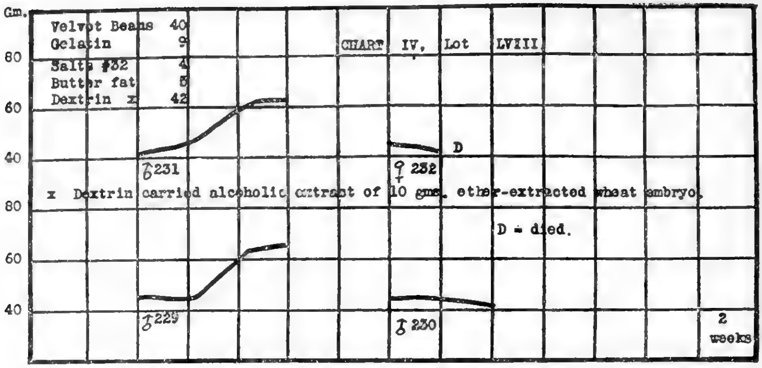

CHART IV, LOT LVIII. The behavior of the animals on this ration certainly indicates that gelatin does not furnish the necessary missing links deficient in the proteins of the velvet bean.

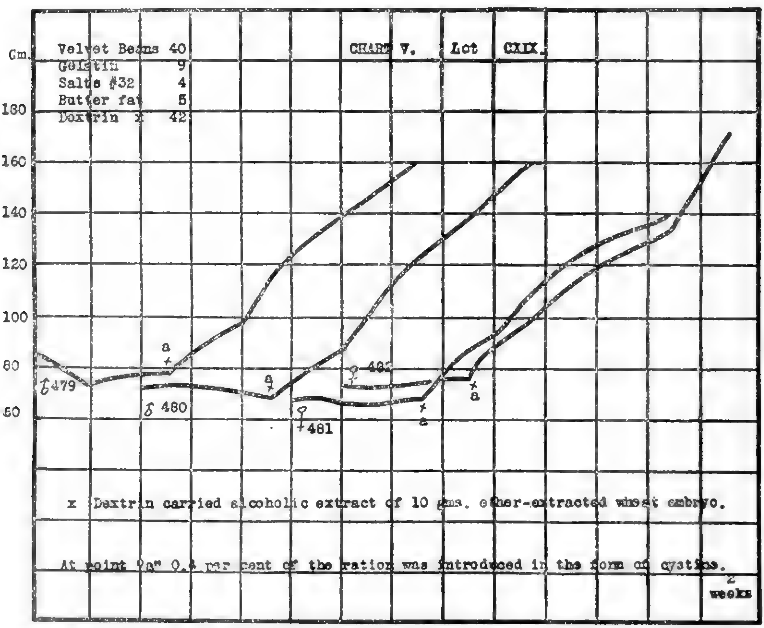

CHART V, LOT CXIX. This is a duplicate of the preceding experiment, Lot LVIII. The experiment was repeated using animals a little heavier, so that they could better maintain themselves on the poor velvet bean-gelatin ration, and in order to have an opportunity to make a cystine addition. After the adding of 0.4 per cent of the ration in the form of cystine,

the writer found, to his great surprise, that all the animals, although they have previously failed to make any growth (Chart IV, Lot LVIII) and produced only maintenance curves on the same ration in this experiment, have begun to grow in a very marked manner, and have made excellent growth for a period of 10 weeks after the amino-acid addition, after which time the experiment was discontinued. This experiment, then, furnishes conclusive evidence that, providing other amino-acids in the form of the deficient protein (gelatin) are supplied, cystine shows itself up remarkably as one of the determining growth-limiting factors in the proteins of the Georgia velvet bean.

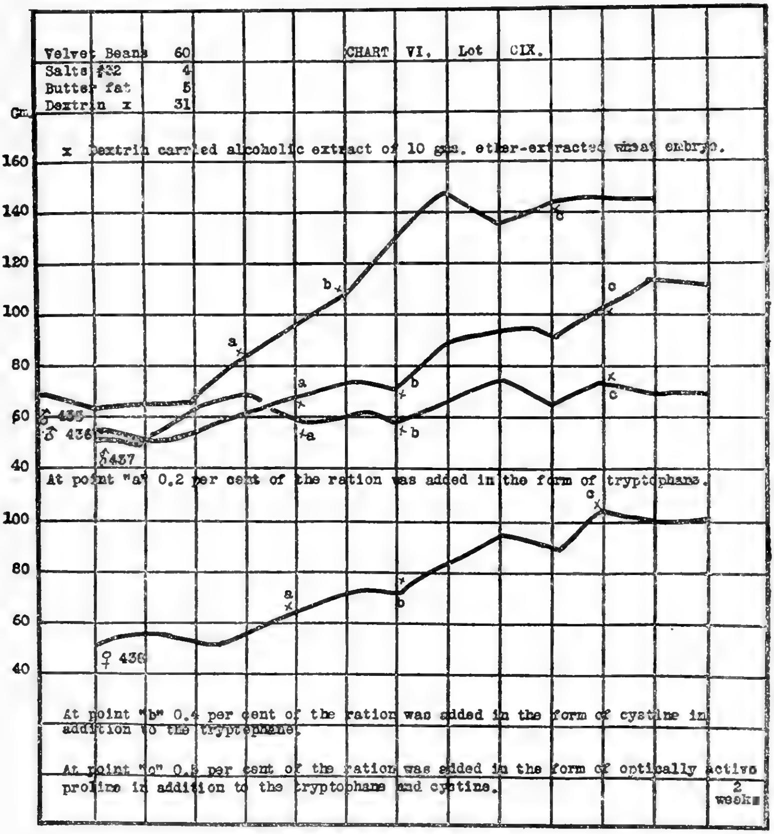

Chart VI, Lot CIX. This ration illustrates that even when the Georgia velvet bean proteins are fed at as high a level as 60 per cent, which would furnish 16.5 per cent protein, very little growth takes place. At point "a" 0.2 per cent of the ration was added in the form of tryptophane, but without any response. At point "b", after the addition of cystine to the extent of

0.4 per cent of the ration, Animals 436 and 438 showed only a slight improvement in growth. Rat 435 shows little change in the slope of the curve, and Rat 437 shows no response at all. It is certainly not convincing from this experiment that cystine is a growth-limiting factor in the proteins in question. The introduction of proline in the ration at point "c" in addition to the tryptophane and cystine brought no response.

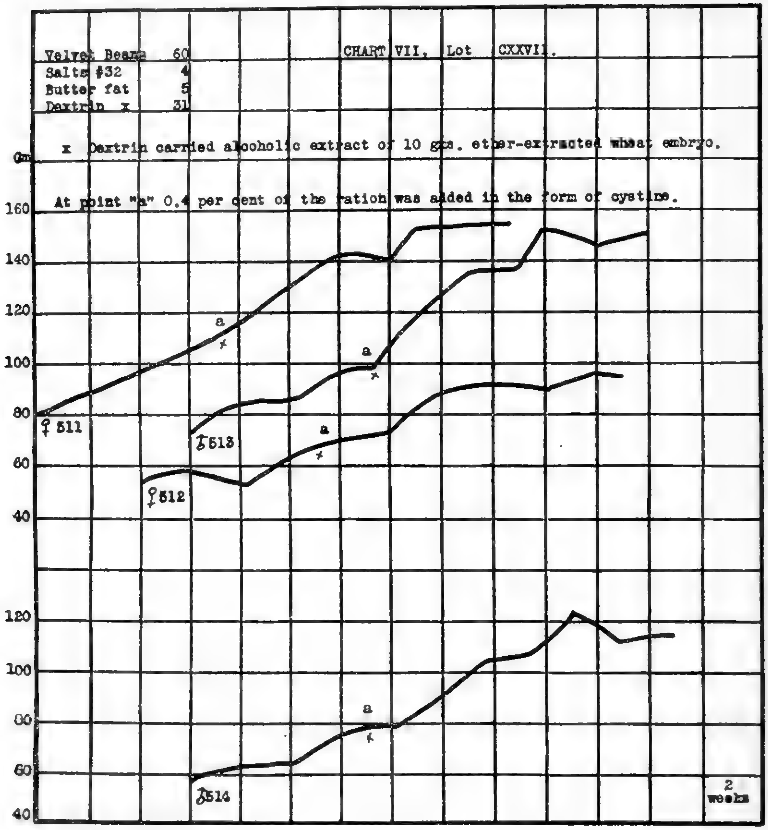

Chart VII, Lot CXXVII. This experiment is a duplicate of the one just preceding, Lot CIX, the purpose being to make the addition of cystine in the absence of tryptophane. There seems to be some response to cystine in the case of Animals 513 and 514, but certainly very little, if any, in the case of Rats 511 and 512. On the whole, judging from the character of amino-acid responses of this lot and the preceding lot, No. CIX, it seems that the suggestive response to cystine takes place to the same extent in the absence of tryptophane.

DISCUSSION.

Previous work (6) on the biological analysis of the seed of the Georgia velvet bean indicated that 40 per cent is the optimum plane of seed intake; therefore, 40 per cent was the level chosen for the study of the quality of proteins of this bean from the standpoint of amino-acid deficiencies. Chart I, Lot LV indicates that the addition of cystine to the proteins in question fed at the optimum level, even in the presence of arachin, brings about no response, and one might conclude from this experiment that cystine is not a growth-limiting factor in the proteins of the Georgia velvet bean. Further work, however, showed that when cystine is added to a 40 per cent level of the seed in the presence of gliadin or zein, definite, although not very marked, improvement in the character of growth takes place. The addition of cystine, however, to a velvet bean-gelatin ration, on which diet only maintenance takes place, is immediately followed by considerable growth which is continuous. The addition of cystine in the presence of the above mentioned deficient proteins was made with the following idea in mind. Picturing the possibility that the proteins of a seed may owe their deficiency to a number of amino-acids as represented by a, b, c, d, e, f, and g, and supposing we are interested to know if *g* representing *cystine*, is a growth-limiting factor; also supposing that a, b, and c, belong to the monoamino group which the animal organism may be able to synthesize readily, then amino-acids d, e, and f will still have to be supplied before a response to *g* may be obtained. This furnishes the basis for the addition of the deficient proteins between the seed and the amino-acid. Evidently gliadin and zein are furnishing some amino-acids in which the proteins of the Georgia velvet bean are lacking but not to the extent that gelatin is; hence, the more marked response in the presence of gelatin.

In a former communication (2) the author stated that Mr. Koehler of the Laboratory of Agricultural Chemistry, University of Wisconsin, very carefully analyzed zein, purified by reextracting three times with 70 per cent alcohol, and could find no cystine in it. On improving his technique for the study of the cystine content of proteins after hydrolysis, Mr. Koehler has found

(7) that a great portion of the organic sulfur of zein is in the form of cystine, also that casein is much higher in cystine than formerly reported. In view of this work, it is possible to see why fairly good growth was obtained during the first 8 weeks on a zein-velvet bean ration, and that later a response to cystine addition was secured.

Although previous work indicated that 40 per cent is the optimum level of velvet bean seed intake, an attempt was made to add cystine to a 60 per cent plane of intake, in order to see if the amino-acids from 20 per cent additional seed could replace 9 per cent of any of the deficient proteins employed in this investigation. It will be noted, however, that while some response is suggestive in the case of certain animals of the duplicate lots, Nos. CIX and CXXVII, the results are not at all conclusive; and the data clearly indicate that without the use of the deficient proteins it would have been impossible to find that cystine is a determining growth-limiting factor in the proteins of the Georgia velvet bean, since Waterman and Jones (3) have failed, just as the writer has in his preliminary work, to secure a response to straight cystine additions to the velvet bean seed.

The addition of tryptophane and proline in the presence of cystine brought about no response.

A problem that suggests itself in connection with this work is: What amino-acids is gelatin furnishing to the proteins of the velvet bean that are essential before a response to cystine can be secured. This study is under investigation and will be reported later.

Preliminary work on amino-acid deficiencies in edestin (the globulin from hemp seed (8)) indicated that certain amino-acids are deficient in that protein, but the work was not at all conclusive. Employing the improved technique described in this paper to explore further the cause of the poor nutritive quality of edestin, the author has obtained corroborative evidence of the earlier suggestive data. The results of the experiments on amino-acid deficiencies in edestin will appear in a following communication.

SUMMARY.

1. The proteins of the Georgia velvet bean are deficient in character.

2. Arachin does not supplement the proteins in question; neither is there a response obtained to the addition of cystine in the presence of arachin.

3. There was no response to the addition of proline even in the presence of cystine and tryptophane.

4. A slow but definite response to cystine was secured in the gliadin-velvet bean and the zein-velvet bean rations.

5. A very marked and continuous response to cystine was obtained in the gelatin-velvet bean maintenance ration.

6. *Cystine is a determining growth-limiting factor in the proteins of the Georgia velvet bean, a fact which becomes apparent only in the presence of such deficient proteins as gliadin or zein, and most markedly in the presence of gelatin.*

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AN ORGANIC CONSTITUENT OF THE TUBE OF MESOCHÆTOPTERUS TAYLORI, POTTS.

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Mesochætopterus taylori is a polychæte worm which is known to occur only on the northwest coast of North America. It has been described in detail by Potts (1). It may be found in the neighborhood of Nanaimo, wherever a stretch of clean sand is exposed at low tide, inhabiting a long tube constructed of thin but tough material in which sand is imbedded.

The writer (2) has recently made pentose determinations in a number of substances of marine animal origin, employing Grund's modification of the method of Tollens and Kröbe (3). Of all those examined the material of which the tube of *Mesochætopterus taylori* is composed gave the highest percentage yield of furfural and the presence of a large quantity of a pentose derivative was suspected. All the pentose compounds which had previously been encountered in animal material were constitutents of organized tissues, were soluble in water, and consisted either of nucleotides or β-nucleoproteins. It seemed *a priori* improbable that a compound of either of these classes should occur in a secreted material like the tube of a worm and their absence was confirmed when the furfural-yielding constituent was found quite insoluble in water.

The nature of this constituent seemed worth investigating. It was subsequently found that more than one furfural-yielding substance was involved. The present communication deals with one of them. It has not been fully characterized, since neither sufficient material nor adequate facilities were available, but there seems little doubt as to the class of compounds to which it belongs.

EXPERIMENTAL.

The animals were carefully removed from the tubes and the tubes, after washing as free from sand and debris as possible, dried first at air temperature and finally at 100°C. More sand was removed from the dry substance by grinding and sieving, but a certain amount remained imbedded in the substance of the tube.

The material thus prepared contained 32.3 per cent of ash and yielded 3.5 per cent of furfural on treatment with 12 per cent hydrochloric acid. 140 gm. (corresponding to 400 to 500 tubes) were covered with a 2 per cent solution of sodium carbonate and kept between 40 and 50°C. for 24 hours. On making the alkaline extract distinctly acid with hydrochloric acid a close grained flocculent precipitate was produced. An excess of alcohol was added and the precipitate filtered off, washed with alcohol, and dried at 100°C. It yielded furfural on boiling with 12 per cent hydrochloric acid, gave slight xanthoproteic and biuret reactions, and was evidently not of the same character as the precipitate obtained from subsequent extractions. It was accordingly held separately from these. Subsequent extractions yielded on acidification a heavy bulky transparent gelatinous precipitate. After addition of alcohol this was easily filtered off through muslin, but, owing to its colloidal nature, could not be washed entirely free from hydrochloric acid. The tube material was extracted five times for 24 hour periods and the precipitates were accumulated in alcohol. The fifth extraction did not yield very much precipitate and the process was discontinued. The residual substance, after washing free from sodium carbonate, and drying, weighed 77 gm.; it contained 24.4 per cent of ash and yielded 2.5 per cent of furfural, so that, either the extraction was less complete than seemed to be the case from the small quantity of material obtained by a repetition of the treatment, or more than one furfural-yielding body was involved. The latter proved to be the case since it was found that such a body could be extracted by 1 per cent sodium hydroxide, after which the residue yielded only a trace of furfural. The substance obtained by neutralization of the sodium hydroxide extract was of an entirely different physical character to that which forms the subject of this paper and its further examination has yet to be made.

The substance precipitated by acidification of the sodium carbonate extract bore a very close superficial resemblance to the "alginic acid" which has been isolated from a number of marine algæ and was at first taken for it or a similar pentosan derivative. It was extractable in the same way and altered in physical properties if extraction were carried out at a higher temperature than 50°C. Like alginic acid it consisted of a colloidal substance capable of absorbing several times its own weight of water, readily soluble in dilute alkali when moist, but becoming hard, horny, and resistant to solvents when dry, forming insoluble salts with the heavy metals, soluble ones with the alkalies, and yielding furfural in considerable quantity on treating with 12 per cent hydrochloric acid. It gave no protein reactions, but contained organic nitrogen.

The method employed for the purification of alginic acid by Hoagland and Lieb (4) was tried on the substance. The crude material was well washed with alcohol, freed from alcohol as far as possible by suction, dissolved in the least possible amount of warm 2 per cent sodium carbonate, and poured into alcohol. This precipitated the sodium compound in the form of non-gelatinous strings drying to a brownish tough horny mass swelling enormously and eventually dissolving in water, precisely like the corresponding salt of alginic acid. 5 gm. of the sodium salt were dissolved in water and dialyzed in a collodion bag in running water for a week. At the end of this time a sample, removed and acidified, yielded material still containing 17.7 per cent of ash and nitrogen. The dialysis was therefore continued for another week, but no appreciable reduction of ash constituents resulted and the nitrogen reaction persisted. The main solution was therefore acidified which produced a solid jelly. This was broken up as far as possible in water and the suspension returned to the dialyzer. After 10 days most of the hydrochloric acid had been removed and only a slightly viscous solution remained which yielded very little precipitate on adding alcohol either before or after acidification. The free acid substance had either decomposed or diffused through the membrane on removal of the bulk of the hydrochloric acid. In the light of knowledge of the composition of the substance subsequently obtained the former is the more likely alternative. In respect of this inability

to purify by dialysis the substance differed essentially from alginic acid.

The remainder of the sodium salt was dissolved in water and the free acid reprecipitated with hydrochloric acid. This was dissolved in 2 per cent sodium carbonate and the sodium salt again precipitated by alcohol. After washing with alcohol and drying at 100°C. it weighed 40 gm., contained 33.04 per cent of ash, and yielded 4.1 per cent of furfural.

Acid Hydrolysis.

The sodium salt yielded a solution with strong reducing action on treating with mineral acids. It was, however, very resistant to acid hydrolysis; 50 hours heating in a boiling water bath with 6 per cent hydrochloric acid was necessary to attain a maximum reducing power. Weaker acid was almost without action. Stronger acid led to excessive carbonization accompanied by production of furfural, and heating beyond 50 hours led to decrease of reducing action.

10 gm. of sodium salt were dissolved in 50 cc. of water and 50 cc. of 12 per cent hydrochloric acid added. After heating in a boiling water bath for 50 hours the solution had a reducing action indicating a yield of 24.4 per cent of reducing sugar (calculated as dextrose). This was far in excess of the reducing action to be anticipated from a pentose calculated from the furfural yield and it was clear that, either the furfural was not produced from a pentose derivative, or some other reducing substance, in addition to the furfural-yielding one, was produced by acid hydrolysis. That the latter alternative was true was shown by the fact that on distilling the substance with 12 per cent hydrochloric acid until no further furfural came over, the residue still had a powerful reducing action. It seemed also true that the production of furfural was not due to a pentose derivative, for, on testing the solution of the fully hydrolyzed substance for free pentose with Bial's orcinol reagent there was no reaction and, on making the acid strength of the solution up to 12 per cent and distilling, no furfural was obtained. The furfural-yielding substance had therefore been destroyed during the hydrolysis.

The only substances known to occur naturally and to yield furfural in considerable quantity on treating with 12 per cent

hydrochloric acid, in addition to pentose compounds, are the oxycelluloses, galacturonic and glucuronic acids, and their derivatives. The first two classes of compounds have hitherto been identified with certainty only in vegetable material. Glucuronic acid seemed therefore to be the most probable furfural-yielding compound to be involved in the substance under discussion. The origin of the substance added to the probability since the worm-tube is undoubtedly constructed from a secretion of a mucoid nature. Chondroitin sulfuric acid and allied compounds have been prepared from various mucoids and, in at least one case, that of chondromucoid, it has been shown by Levene and La Forge (5) that the chondroitin sulfuric acid contains glucuronic acid as one of its components. Its physical properties, its resistance to acid hydrolysis, and the simultaneous destruction of the furfural-yielding residue under the treatment, also point to a compound of the nature of chondroitin as the source of the furfural. Since the component believed to be glucuronic acid was broken down by ordinary methods of hydrolysis and sufficient material was not available to attempt the method used by Levene and La Forge to isolate it from chondroitin, glucuronic acid has not been positively identified. Its presence is inferred from the foregoing argument and the identification of other constituents similar to those of chondroitin.

Preparation of Phenylosazone.

The remainder of the solution resulting from the acid hydrolysis was boiled with a little animal charcoal and filtered. The filtrate was neutralized with sodium hydroxide, evaporated to small volume, and poured into about 5 volumes of alcohol which threw down a resinous substance. The solution was separated from this and evaporated, the residue taken up with alcohol, again evaporated, and finally taken up with about 25 cc. of water. 1.5 gm. of phenylhydrazine hydrochloride, 2.5 gm. of sodium acetate, and 2.5 cc. of glacial acetic acid were added and the whole was heated in a boiling water bath. After heating for 2 hours only a small quantity of black resinous material had separated. On filtering this off and cooling the filtrate a small quantity of a fine crystalline precipitate formed. This was filtered off and the mother liquor heated for another hour, which led to fur-

ther precipitation. Finally, on concentrating the liquor somewhat, a third crop of crystals was obtained. The first fraction was recrystallized from hot water and, after drying, melted at 173–174°C. After a second recrystallization with a little alcohol present it melted at 178–179°C. The second fraction, after recrystallization, melted fairly sharply at 179°C. and the third at 182–184°C. It seemed, therefore, that all three fractions consisted in the main of the same compound and that its melting point was somewhere between 179 and 184°C. This is very near the melting point (180–185°C.) of the osazone originally obtained by Levene and La Forge (6) from chondrosamine derived from chondroitin, though in a later paper Levene (7) shows that, if sufficiently purified, their osazone melts at 201°C. and is identical with galactosazone.

Levulinic Acid.

10 gm. of material were heated in a boiling water bath with 100 cc. of 18 per cent hydrochloric acid for 22 hours under a reflux condenser. After boiling to insure freedom from furfural the solution was filtered and extracted four times with ether. The ethereal extracts were combined and evaporated leaving a small brown syrupy residue which was heated gently until free from volatile acid and dissolved in a little water. The solution gave a good iodoform reaction on adding a drop to a warm alkaline solution of iodine. Zinc oxide and a little animal charcoal were added and the solution was boiled and filtered. On standing a small quantity of crystalline salt separated. This was filtered off, washed with alcohol and ether, and dried. Slight charring took place on drying. The dry salt dissolved only partially on boiling with water, leaving a brown residue. Addition of silver nitrate to the solution produced a small precipitate which dissolved on boiling, with formation of a little silver oxide. The filtrate from this on standing in the dark deposited a crystalline precipitate, but the amount was too small to characterize further.

The presence of levulinic acid in the solution, and thus of a hexose or hexose derivative in the original substance, is indicated by the above tests. Levene and La Forge (8) point out that a good yield of levulinic acid can be obtained from chondrosin only after previous deamination. Omission of this preliminary treatment probably accounts for the small amount obtained.

Attempt to Isolate Hexosamine.

The presence of an amino compound was indicated by the fact that the solution of the substance evolved ammonia very readily on warming with sodium hydroxide and contained no protein or ammonium salt. It was confirmed by obtaining nitrogen on treating the solution with acetic acid and sodium nitrite. An attempt was made to isolate the amino compound as hydrochloride. 5 gm. of the sodium salt were ground as finely as possible and boiled with 25 cc. of concentrated hydrochloric acid until furfural evolution ceased and nothing but a small quantity of charred material remained undissolved. This was filtered off, and the filtrate decolorized with animal charcoal and evaporated to small volume. Nothing separated on standing. On pouring into a large volume of alcohol a white amorphous precipitate separated. This was filtered off, washed carefully with alcohol and ether, and dried in a desiccator. It dried to resinous lumps readily soluble in water. The solution reduced strongly evolved ammonia on treating with sodium hydroxide and gave a phenylosazone, but a crystalline hydrochloride could not be separated from it. Levene and La Forge (8) mention the difficulty of obtaining the hexosamine hydrochloride from chondroitin sulfuric acid using the crude sodium salt and were successful only when employing a purified barium salt. Unfortunately the relation of the substance under investigation to chondroitin sulfuric acid did not become apparent until most of the raw material was used up and the height of the tides made it impossible to obtain more.

Detection of Sulfuric and Acetic Acids.

The aqueous solution of the substance gave a colloidal precipitate of the barium salt of the complex acid on addition of barium chloride. After treating the solution with hydrochloric acid barium sulfate was precipitated on addition of barium chloride. It was clear, therefore, that the compound contained organically combined sulfuric acid.

The detection of acetic acid was less certain. On distilling 5 gm. of material with 50 cc. of 25 per cent sulfuric acid a strongly acid distillate was obtained. The distillation was continued,

keeping the volume constant in the reaction flask meanwhile by additions of water, until the distillate was only slightly acid. The distillate was neutralized with sodium hydroxide and evaporated to dryness. The residue gave doubtful cacodyl and ethyl acetate reactions, but unquestionably consisted of a salt of an organic acid.

CONCLUSION.

The substance extracted from the tube of *Mesochætopterus taylori* by warm 2 per cent sodium carbonate has thus been shown to contain, besides a furfural-yielding substance, probably glucuronic acid, a hexosamine, probably galactosamine, sulfuric acid, and a volatile organic acid. It thus bears a close resemblance to the chondroitin sulfuric acid from chondromucoid investigated by Levene and La Forge. To determine whether it is identical with that compound a great deal more material would be necessary.

Furfural has been obtained in approximately the same proportion as is yielded by the tube of *Mesochætopterus taylori* from the tubes of a local *Spiochætopterus* and *Sabella* and from that of *Chætopterus variopedatus*. It is likely, therefore, that similar compounds exist in the tubes of these worms. A complex of like nature seems to have been described by Kelly (9) from the tube of *Spirographis*. It is therefore probable that the occurrence in worm-tubes is general.

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CURVE OF SUGAR EXCRETION IN SEVERE DIABETES.

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The work reported in this paper represents a study of the total quantities of sugar excreted in the urine of a number of diabetic patients during periods in which they were being held in the non-diabetic status, or what is sometimes called the "sugar-free" condition, by dietary restriction, and during the periods in which the diets were gradually increased until clearly abnormal quantities of sugar appeared in the urine. The purpose has been to plot the curves of glucose excretion by diabetic patients on diets increasing gradually from those on which they show no abnormal glycosuria up to the point at which definitely abnormal glycosuria is induced. The total sugar excreted in the urine every 24 hours was determined quantitatively by the method described by Benedict and Osterberg[1] for the quantitative determination of sugar in normal urine.

The glucose equivalents of the diets were calculated as the utilizable carbohydrate plus the protein $\times$ 0.58 plus the fat $\times$ 0.1 as per the equation $G = C + 0.58P + 0.1F$[2] in which G = glucose, C = carbohydrate, P = protein, and F = fat. The total glucose supply to the organism was not always represented entirely by the diet since in most instances the diets were at times below maintenance values, necessitating the catabolism of protein and fat from the tissues. In some instances also, the quantity of fat in the diet could have exceeded the quantity actually catabolized.

Case 1.—(Miss R.) Age 60 years. Weight 38.6 kilos. On admission to the hospital she showed marked denutrition, glycosuria, acidosis, alkali deficit, and symptoms of acid intoxication. She was given alkali and

[1] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

[2] Woodyatt, R. T., *Arch. Int. Med.*, 1921, xxviii, 125.

reduced to the non-diabetic status by dietary restriction. On a diet of 32 gm. carbohydrate, 57.5 gm. protein, and 53 gm. fat—containing 835 calories and the equivalent of 71 gm. glucose (G), the total sugar eliminated each 24 hours for 3 days remained constantly at a little over 400 mg. per day (see Chart 1). The diet was then increased very slowly; and on 35 gm. carbohydrate, 60 gm. protein, and 60.5 gm. fat—924 calories (G = 76), the sugar excreted per day for 2 days was no higher than it had been on the 835 calorie diet. On the 3rd day, however, while the patient was still on the same diet, the sugar eliminated suddenly increased to 1,050 mg. and a subsequent addition equivalent to 4 gm. glucose and 34 calories resulted

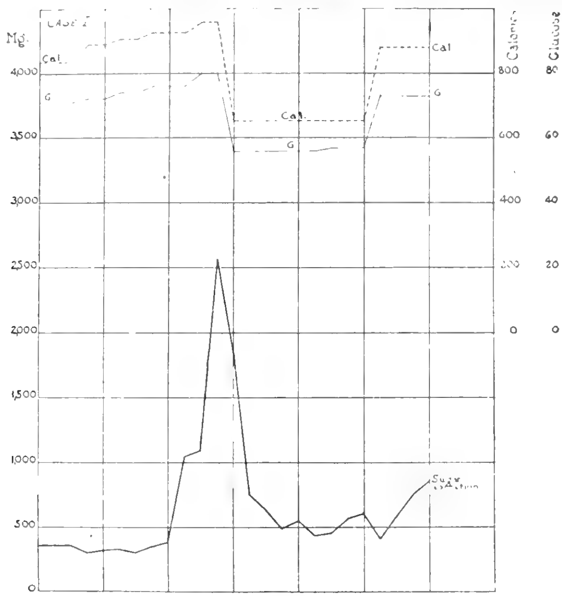

Chart 1.

in the excretion of 1,092 mg. for the first 24 hours and 2,556 mg. for the second 24 hours on which the same diet was maintained. Without entering into a discussion of the factors which may have determined this sudden acceleration of the glucose excretion in this particular case, it affords an example of the suddenness with which the sugar excretion may jump from a normal level to an abnormally high level.

Case 2.—(Mr. M.) A young man, 26 years of age, weighing 50 kilos, who entered the hospital in severe acidosis and denutrition and who had been "desugarized" by a diet consisting of green vegetables and broth aggregating 298 calories (G = 27), excreted 520 mg. of sugar on the first day of observation. Thereafter, the sugar excreted in the urine remained

practically constant from day to day during the time in which the diet was increased from a caloric value of 298 to one of 1,336 calories, the G for the diet rising from 27 to 116 gm. Thus, the total quantity of sugar eliminated for the 24 hour period in which the diet contained 398 calories with $G = 51$, was 680 mg. and it was only 683 mg. on a 1,236 calorie diet with $G = 101$ gm. The next addition, which brought the diet up to 1,404 calories, and increased G to 123 gm. (an increase of 22 gm.) resulted in a total urinary sugar excretion of only 1,006 mg.; whereas, upon a further addition which increased the value of G by only 11 gm. and the calories by 50, the total

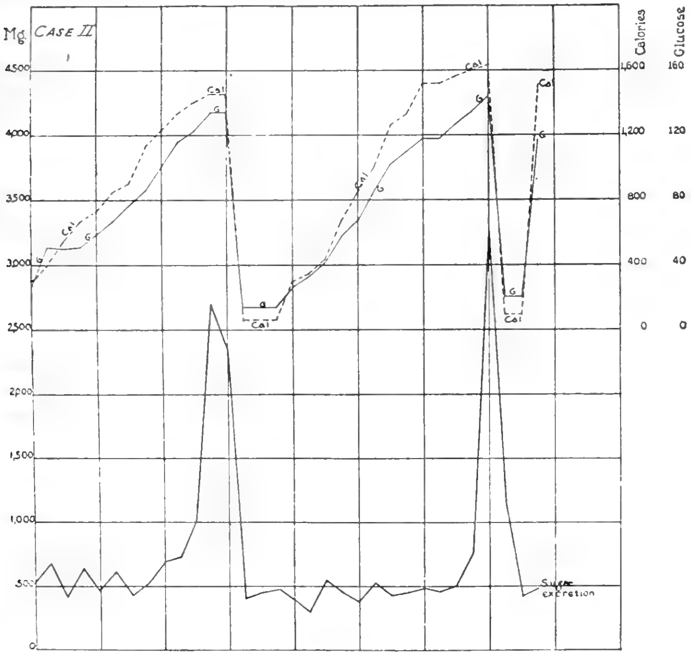

CHART 2.

sugar eliminated was 2,695 mg. Thus, increasing the calories in the diet by some 800 calories and the glucose equivalent by 89 gm. caused little or no increase of the sugar excretion, but the further addition of only 218 calories and the equivalent of 33 gm. glucose caused a critical break in the curve. On the lowest diet the calories were 298; therefore most of the food supply on which the patient was subsisting was then coming from the tissues. The highest diet barely approached maintenance requirements. Accordingly, the figures for the diet never represented the total glucose supply from all sources, and the curve of increase of the latter is not known. Yet the experiment shows a sudden transition from the normal

to the abnormal in respect to the sugar excretion. The diet was then reduced to 64 calories with *G* for the diet itself at 14; and the experiment was repeated with the same result. There was almost no variation in the total sugar eliminated per day during the time that the diet was increased from 14 *G* and 64 calories to 135 *G* and 1,601 calories; whereupon, a subsequent addition of food equivalent to only 9 gm. glucose and 36 calories, which brought *G* for the total diet to 144 gm. and the calorie value to 1,637, resulted in a sugar excretion of 3,268 mg. for that day. It was quite evident that the curve representing the sugar eliminated in the urine by

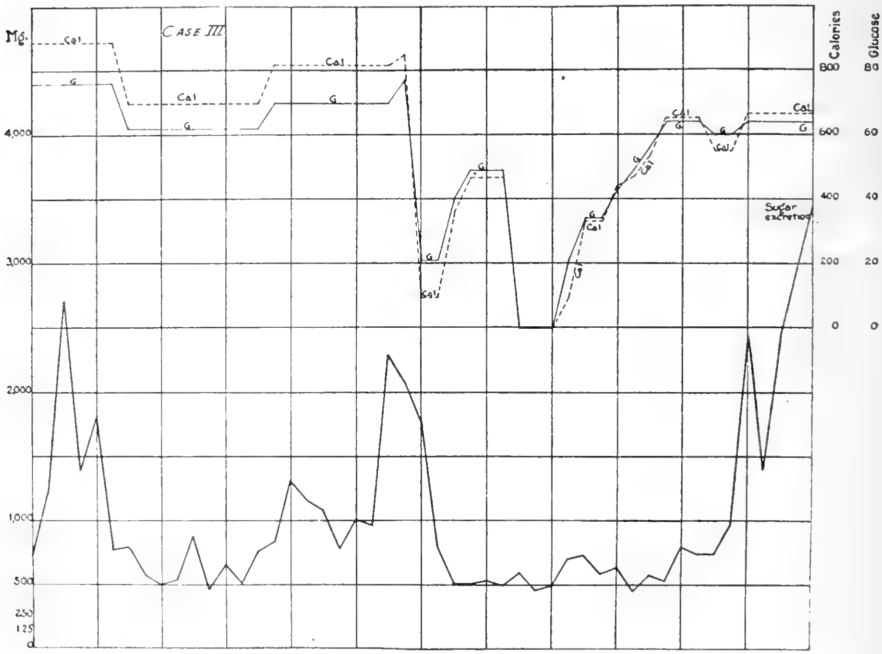

CHART 3.

days, did not follow the curve representing the gradual rise in the caloric or glucose equivalents of the diet. It remained more or less constant until the glucose equivalent of the diet reached a certain value, when there was a sudden upward break in the curve of sugar excretion. This upward break represented an absolute acceleration of the sugar excretion out of proportion to that which had resulted from all earlier additions to the diet. It also represented an even greater increase of the percentage excretion of the last increments to the diet.

Case 3.—(Mrs. F.) A young woman 24 years of age. Weight 36 kilos. Her glucose-using power was very low. On an 888 calorie diet (with

G = 76 gm.) she excreted 725 mg. sugar on 1 day, and 2 days later, while still on the same diet, excreted 2,711 mg. On absolute starvation for 3 consecutive days her urinary sugar excretion was 601, 460, and 504 mg. per day. On a 651 calorie diet, with G = 64 gm., the urinary sugar was 537, 802, and 750 mg., respectively for the 3 days on which the same diet was maintained; whereas, a slight subsequent rearrangement in the diet, increasing its calorie value only to 665, with G as before, resulted in the excretion of 2,440 mg. sugar. Here again, the transition from the "sugar-free" state to one in which the urine contained distinctly

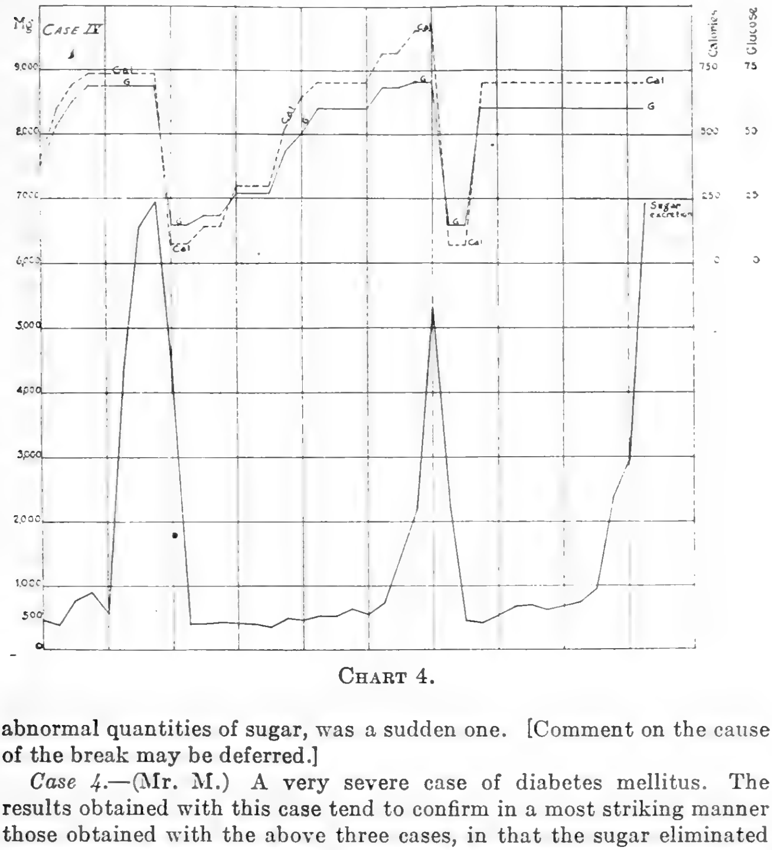

Chart 4.

abnormal quantities of sugar, was a sudden one. [Comment on the cause of the break may be deferred.]

Case 4.—(Mr. M.) A very severe case of diabetes mellitus. The results obtained with this case tend to confirm in a most striking manner those obtained with the above three cases, in that the sugar eliminated in the urine did not increase uniformly with the gradually increasing diet; but that there was a critical point at which a sudden and abnormally high excretion of sugar occurred in the urine.

Case 5.—(Mr. C.) Age 42 years. Weight 52 kilos. This patient's diet was built up gradually from 415 calories with G = 36 gm. to 1,295 calories

with G = 107 gm., while the total sugar eliminated in the urine every 24 hours remained practically constant (except on 1 day following a day of low excretion). In this case the diet at no time reached the point where a real critical acceleration of the sugar excretion occurred. The sugar excreted on the diet of 1,295 calories with G = 107 gm. was no higher than that excreted on the diet of 415 calories, for which G was 36 gm. This patient was neither in caloric nor nitrogenous equilibrium on the diet given, but left the hospital to continue treatment at home so that the chart was interrupted

Case 6.—(Mr. A.) A poorly nourished young man of 25 years. Weight 44.1 kilos. A severe case of diabetes mellitus. This patient came into the hospital with much sugar, acetone, and diacetic acid in the urine, and

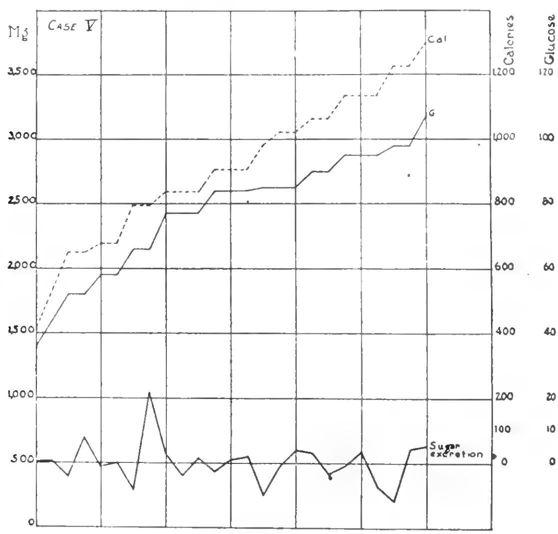

CHART 5.

a marked alkali deficit in the blood. He did not "desugarize" on a diet consisting of 400 gm. of "greens" and 1 liter of broth even after 4 days; and was fed more liberally till the acetone and diacetic acid disappeared. Then, following several dietary manipulations, his urine no longer gave a qualitative test for sugar. On a diet of 953 calories, for which $G = 88$ gm., the total sugar elimination was between 400 and 500 mg. per day; and on a diet of 1,837 calories, with $G = 107$ gm., the sugar excreted in the urine was only 330, 486, and 333 mg.

While the total quantity of sugar eliminated per day on a diet of 64 calories, with G at 14 gm. was practically the same as that eliminated on a diet of 1,993 calories (G = 114.7), it jumped suddenly to 1,485 and 9,800 mg. respectively when the diet was increased to contain 2,080 calories and the

equivalent of 121 gm. glucose. In this case observations were made of the relative effects of a high fat, high carbohydrate, low protein diet; and a high fat, high protein, low carbohydrate diet, each having the same value for *G*. These diets sufficed for maintenance. When the *G* of the diet was kept below a certain value, there was no significant change in the total urinary sugar excreted from day to day, regardless of changes in the relative quantities of carbohydrate and protein. In this case working laterally with maintenance diets, endogenous factors were reduced to a minimum.

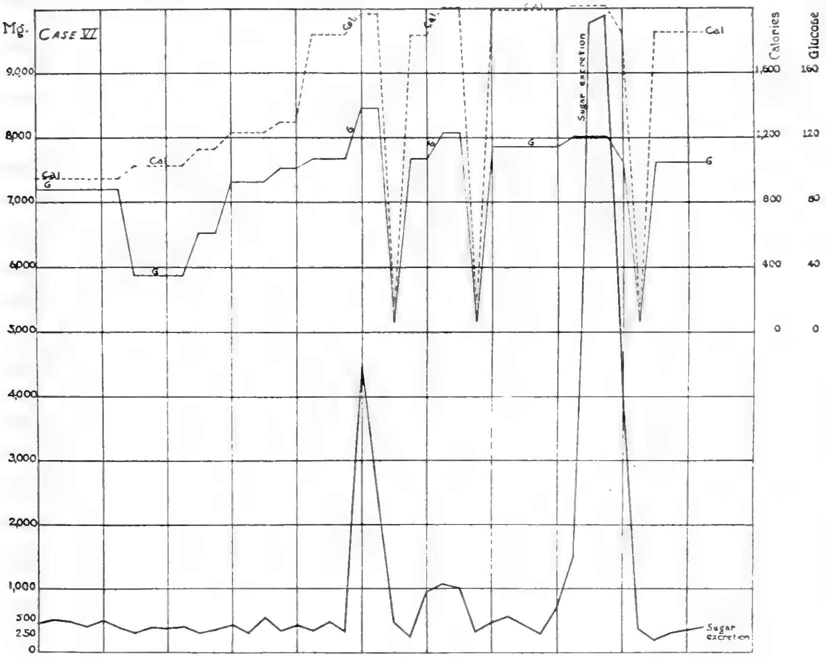

CHART 6.

Case 7.—(Mrs. L.) Age 31 years. Weight 40 kilos. The experiment was begun with the patient on a diet of 830 calories, with $G = 49$. In this case the additions to the diet were made uniform. She was first kept on a constant diet of 25 gm. carbohydrate, 30 gm. protein, and 67 gm. fat, consisting of 400 cc. 5 per cent vegetables, 24 cellulose muffins, 3 eggs, 15 gm. butter, 100 cc. cream, 30 gm. bacon, and 10 gm. rice. For 11 days the sugar excretion was charted while the diet remained the same. During this period the excretion was not at first constant but it became so for the last 5 days (average 562 mg.). Thereafter additions were made always in the form of cream containing 16 per cent fat, with no other changes. The first addition consisted of 50 cc. cream. 3 days later, 25 cc. cream were

added and thereafter 25 cc. more each 4th day until six additions had been made, then 25 cc. cream every other day. In this case each fresh addition to the diet tended to cause a slight increase of the sugar excretion for that day followed by a return to the former level on the following day. These fluctuations were most marked early in the experiment and grew less as time passed. The average excretion was very constant at 600 to 700 mg. While no change took place in the total quantity of sugar eliminated per day (500 to 1,000 mg.), during the time that the glucose equivalent of the diet rose from 49 gm. to 70.3 *G* and the calories from 830 to 1,270, there was a sharp break in the sugar eliminated with the next addition of 25 cc. cream. With the diet aggregating 1,270 calories with $G = 70.3$ gm. the excretion ran 942 and 1,420 mg. on 2 successive days. The next addition was followed by excretions of 1,504 and 1,906 mg. Another addition was

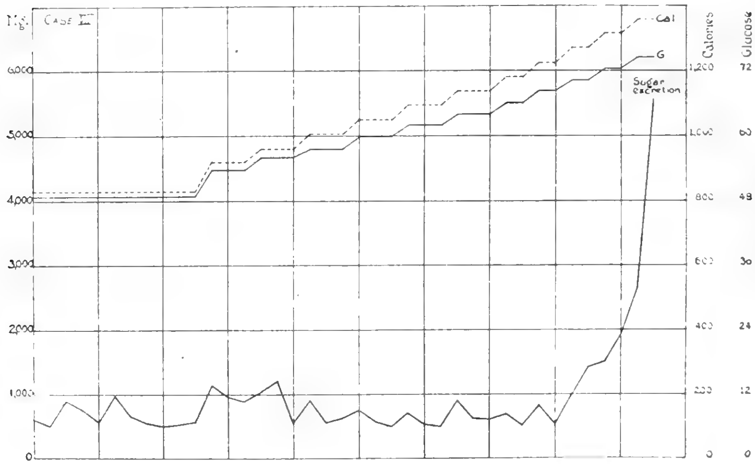

CHART 7.

then made and the excretion rose to 2,650, 5,568, and 6,300 mg. In this case it will be noted that when the total glucose equivalent of the diet was 68 gm. the excretion was still only 825 and 529 mg. on 2 days. The subsequent addition of food equivalent to 6.3 gm. glucose led to the excretion of approximately this quantity of glucose over and above the former average. There was, in short, a virtually complete excretion of all the glucose supplied in excess of a certain limit.

CONCLUSION.

Study of the curves obtained leads to the conclusion that individuals with severe diabetes, when brought into the non-diabetic status (or, as it is sometimes called, the "sugar-free" state) by

fasting or other more suitable adjustments of the diet, may then excrete small quantities of sugar not greater than those excreted by normal individuals under parallel conditions. The quantities in this series averaged between 10 and 15 mg. per kilo per day. As the diet is gradually increased stepwise at 1 to 4 day intervals, there is at first little or no *permanent* increase of the sugar excretion. The sugar excreted has remained entirely unaffected; or it has shown a definite but temporary acceleration with each new addition to the diet to be followed by a restoration of the former level; or it has shown a slight rising tendency from the start. But in any case the total permanent increase of the sugar excretion has remained slight or even unrecognizable until the total glucose equivalent of the diet has risen above a certain limit (which varies with the individual). Once this limit has been passed, further additions to the diet lead to rapid—even sudden—accelerations of the sugar excretions, out of proportion to any which have occurred before. The curve may then bend rapidly upward or show a true critical break.

This observation is in harmony with the well known conception of a clearly definable "tolerance limit" for glucose in diabetes; and that an "abnormal" sugar excretion may develop with critical suddenness when this limit is overstepped.

STUDIES ON PROTEINOGENOUS AMINES.

XII. THE PRODUCTION OF HISTAMINE AND OTHER IMIDAZOLES FROM HISTIDINE BY THE ACTION OF MICROORGANISMS.

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CONTENTS.

INTRODUCTION.

In a series of papers published in 1919[1] we communicated a method for the microchemical colorimetric estimation of imidazole derivatives and for the quantitative separation of histamine from histidine. These methods enabled us to study the metabolism of histidine under various conditions. Experiments

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521, 539.

with a colon bacillus, isolated from a case of cystitis, gave results that led us to the following conclusions:

1. When the colon bacillus is allowed to metabolize histidine, either alone or in the presence of nitrates or ammonium salts, histamine is not formed.

2. In a medium containing histidine and glycerol, but no nitrates or ammonium salts, histamine is not formed. In this case imidazole propionic acid appears to be formed; but only when the bacillus is forced to grow anaerobically.

3. In a medium containing histidine, glycerol, or glucose and a source of nitrogen, either KNO_3, NH_4Cl, or both, about 50 per cent of the histidine is converted into histamine in the course of 2 weeks when oxygen is present. In the absence of atmospheric oxygen, this and all the other metabolic activities of the bacillus are greatly reduced, probably because the colon bacillus is an aerobic organism by preference.

4. The production of histamine is always coincident with the production of a medium that is distinctly acid. We believe that the histamine is formed by the bacillus to neutralize the excess of acidity that is simultaneously produced from the glycerol.

5. Contrary to the statement sometimes given in text-books and in the literature that carbohydrates prevent the formation of histamine from histidine, we have found that histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose.

Having established the above facts for one particular colon bacillus, we were then led to a consideration of the following queries:

1. Are all strains of colon bacilli capable of decarboxylating histidine in our standard medium?

2. Are other organisms capable of converting histidine into histamine under identical conditions?

3. How does the addition of amino-acids or peptones to our standard medium containing histidine, influence the production of histamine?

Procedure.[2]

In every case the same number of microorganisms—nine billion[3]—was introduced into 200 cc. of an autoclaved medium

[2] For a detailed description of the method see Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539–555.

[3] The method employed in counting the organisms was that of Breed and Brew (Breed, R. S., and Brew, J. D., *New York Agric. Exp. Station, Techn. Bull. 49*, 1916).

having the following composition: 0.2000 gm. histidine dichloride, 0.2000 gm. ammonium chloride, 0.1000 gm. potassium nitrate, 0.4000 gm. potassium dihydrogen phosphate, 0.800 gm. sodium chloride, 0.0200 gm. sodium sulfate (anhydrous), 0.4000 gm. sodium bicarbonate, 0.0100 gm. calcium chloride (anhydrous), and 4.00 cc. glycerol dissolved in sufficient distilled water to give a final volume of 200 cc.

The inoculated flasks were then incubated at 37° for 14 days, unless otherwise specified, after which the mixture was forced through a Berkefeld filter. The hydrogen ion concentration of the filtrate was determined colorimetrically (see below for details). The filter was then carefully washed with at least 200 cc. of water. Concentrated sulfuric acid (1.0 cc.) was added to the combined filtrate which was then freed from water by evaporation in a glass dish on the water bath. The syrupy residue was transferred, with distilled water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. Of this test liquid 10 cc. were transferred to a 35 cc. glass-stoppered bottle, treated with 3 gm. of solid sodium hydroxide, and extracted six times with amyl alcohol using 20 cc. for each extraction. This divides the material into two fractions, the amyl alcohol extract, which may contain histamine and methyl imidazole and which we refer to as the histamine fraction, and the alkaline aqueous liquid which contains histidine and may contain imidazole acetic, propionic, lactic, and acrylic acids.

The combined amyl alcohol extracts were extracted with normal sulfuric acid, which removes the imidazoles. The acid extracts were nearly neutralized with 5 N NaOH and the resulting liquid was diluted to exactly 100 cc. The amount of histamine present was then determined colorimetrically by means of the well known reaction that occurs between *p*-phenyldiazonium sulfonate and imidazole derivatives in a solution rendered alkaline with sodium carbonate.[1] When the presence of histamine was indicated by the colorimetric determination, an amino nitrogen determination was also carried out on this fraction. The values obtained by these methods check closely when histidine is the only amino-acid present in the original medium. In other cases the amino nitrogen values are invariably high. To be certain that histamine was the only imidazole present, a methyl imidazole

determination was also carried out. We have never encountered methyl imidazole as a product of the bacterial decomposition of histidine. The presence of histamine was, moreover, qualitatively verified by means of physiological methods.

The histidine fraction was transferred to a 25 cc. graduated precision cylinder with water and 7 cc. of 37 per cent HCl. The cooled acid liquid was then diluted to 25 cc. Portions of this liquid were than tested for histidine, colorimetrically and by means of a Van Slyke amino nitrogen determination. When the values obtained check closely, histidine is probably the only imidazole present. If the color obtained is too red, and the colorimetric determination indicates the presence of considerably more imidazole than can be accounted for as histidine by the amino nitrogen method, the excess color is probably due to imidazole acetic, propionic, lactic, or acrylic acids. Which of these acids is present cannot be determined without an isolation experiment. We have made no effort to isolate these acids but have calculated the excess imidazole value as imidazole propionic acid. When the amino nitrogen determination indicates the presence of considerably more histidine than can be accounted for colorimetrically, we are confronted with two possibilities:

1. Some of the introduced ammonia may have been converted into a carboxylated, alkali-stable, primary amino compound—possibly an amino-acid—which would give off nitrogen with nitrous acid; or

2. Some of the histidine may have suffered a rupture of the imidazole ring with the liberation of free amino groups.

Up to the present time we have brought no absolute proof that either of these is the correct explanation for the facts. At present we are inclined to believe that the second of these possibilities is the most probable and our reason for this belief is outlined in the following pages.

Determination of the Hydrogen Ion Concentration.

In our earlier work we employed the set of standard phenolsulfonephthalein tubes furnished by Hynson, Westcott and Dunning to determine the pH of our media after incubation. This set is inadequate for work of this kind because it is useful only

within the narrow limits of pH 6.6 to 8.6. Most of our final media were too strongly acid to fall within the range of this indicator. We have, therefore, prepared a series of standard tubes with a lower pH value of 1.2 by following the method of Clark and Lubs.[4] The indicators selected were phenol red, range 8.6 to 6.6; brom-cresol purple, range 6.8 to 5.2; methyl red, range 6.0 to 4.4; brom-phenol blue, range 4.6 to 3.0; and thymol blue, range 2.8 to 1.2.

We have found that the buffer solutions containing the sulfonephthalein derivatives can be kept for at least 2 years, and hence probably indefinitely, in a sealed Pyrex glass tube after preservation with thymol. It is possible, therefore, to prepare a permanent set of tubes, similar to those distributed by various concerns, for the phenol red range, for all of the sulfonephthalein derivatives. Methyl red, which is not a sulfonephthalein derivative, deteriorates so rapidly that a set of permanent tubes containing this indicator cannot be prepared in the usual way. We have found, however, that the colors produced with methyl red can be imitated by means of an aqueous solution containing mixtures of Congo red and methyl orange. These thymol-preserved aqueous solutions containing Congo red and methyl orange can then be sealed up and kept in Pyrex glass tubes similar to those used for the other standard sets. They seem to keep indefinitely.

The determinations were carried out as follows. A drop of the filtrate to be tested was transferred to a porcelain test plate and mixed with 1 drop of indicator. This procedure was repeated until a color was obtained that was within the range of one of the indicators. Then 1.0 cc. of the filtrate to be tested was mixed with 0.10 cc. of the proper indicator in a Pyrex test-tube and the color compared with that of the standard tubes. The inherent color of the filtrates was never sufficiently intense to have any effect upon the accuracy of the determination.

Method and Table for the Estimation of Small Amounts of Imidazole Lactic Acid.

In our previous papers[1] we reported tables by means of which colorimetric readings could be converted into milligrams of his-

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.

tidine, histamine, methyl imidazole, and imidazole acetic and propionic acids. It seemed desirable to have a similar table for the estimation of imidazole lactic acid. This substance was, therefore, prepared using the method described by Fränkel.[5] The perfectly white solid obtained by this method, after two recrystallizations from water, had the following properties.

1. Melting point 217°.
2. Chlorine—none.
3. Residue on ignition—none.
4. Ammonia—none.
5. Amino nitrogen (Van Slyke method)—none.
6. The solid—0.1000 gm.—was dissolved in 10 cc. of 0.10 N NaOH and allowed to react for 1 hour at room temperature. The excess of alkali was determined by titration with 0.10 N HCl, using phenolphthalein as indicator. The first change in the indicator was obtained when 4.3 cc. of the 0.10 N acid had been added, 4.5 cc. of the acid being required to give a colorless solution. The indefinite end-point obtained is exactly what one would expect of a substance having a fairly strong acid group and a feebly basic group. The 5.7 cc. of 0.1 N NaOH used for 0.1000 gm. of substance agrees very well with the 5.74 cc. demanded by theory for $C_6H_8N_2O_3 \cdot H_2O$. We therefore considered the substance to be 100 per cent pure.

A stock solution was prepared by dissolving 0.5000 gm. of the solid in 28.7 cc. of 0.10 N HCl and diluting with water to 50 cc. From this the standard test solution was prepared by diluting 1 cc. to 100 cc. in a volumetric flask. The tabular values were then obtained by mixing different amounts of this standard solution with the alkaline *p*-phenyldiazonium sulfonate reagent as previously described.[1] The color produced was then compared in a Duboscq colorimeter with a standard solution of Congo red.[6]

The color produced matches that of the Congo red solution perfectly. A color of maximum intensity is obtained within 3 to 5 minutes and it is stable for from 5 to 10 minutes during which time an accurate comparison can easily be made. With

[5] Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 229.

[6] To prepare the Congo red solution, vacuum-dried Grubler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volume flask. Water is then added to the mark. This is the stock solution which keeps indefinitely. From it the standard indicator solution is prepared by diluting 1.00 cc. with distilled water to 500 cc. in a volume flask.

TABLE I.

Estimation of Small Amounts of Imidazole Lactic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole lactic acid ($C_6H_8N_2O_3 \cdot H_2O$) in the test cylinder. (Total volume 8 cc.) Test cylinder set at 20 mm.
mm.	*gm.*
1.0	0.000001
2.0	0.000002
3.0	0.000003
4.1	0.000004
5.1	0.000005
6.1	0.000006
7.1	0.000007
8.1	0.000008
9.2	0.000009
10.2	0.000010
11.2	0.000011
12.2	0.000012
13.2	0.000013
14.3	0.000014
15.3	0.000015
16.3	0.000016
17.3	0.000017
18.3	0.000018
19.4	0.000019
20.4	0.000020
21.4	0.000021
22.4	0.000022
23.4	0.000023
24.5	0.000024
25.5	0.000025
26.5	0.000026
27.5	0.000027
28.5	0.000028
29.6	0.000029
30.6	0.000030
31.6	0.000031
32.6	0.000032
33.6	0.000033
34.7	0.000034
35.7	0.000035
36.7	0.000036
37.7	0.000037

TABLE I—*Concluded.*

Depth of indicator solution (GR) required to match the color in the test cylinder.	Imidazole lactic acid ($C_6H_8N_2O_3.H_2O$) in the test cylinder. (Total volume 8 cc.) Test cylinder set at 20 mm.
mm.	*gm.*
38.7	0.000038
39.8	0.000039
40.8	0.000040
41.8	0.000041
42.8	0.000042
43.8	0.000043
44.9	0.000044
45.9	0.000045
46.9	0.000046
47.9	0.000047
48.9	0.000048
50.0	0.000049
51.0	0.000050

quantities of this acid in excess of 0.000025 gm., the color produced is too intense to enable one to make an accurate comparison. In such cases the test cylinder was set at 10 instead of at 20. The reading obtained was then multiplied by two before subtracting the correction blank, which is 0.30 mm. as in the case of the other imidazoles.[7]

PART I.

On The Products Formed From Histidine by the Action of Bacillus coli.

These four organisms (*Bacillus coli communis, Bacillus coli communior, Bacillus lactis aerogenes*, and *Bacillus acidi lactici*) comprise the colon group (in the narrower sense) according to the classification of the American Public Health Association. This differentiation is based upon the different behavior of these four organisms toward dextrose, lactose, dulcitol, and saccharose and is represented as follows:

[7] This correction blank represents the amount of color that is autogenously produced by the reagent even when imidazoles are absent. See foot-note 1.

Bacillus coli Group.

Dextrose +
Lactose +

Dulcitol +		Dulcitol −	
B. communior		*B. aerogenes*	
B. communis		*B. acidi lactici*	
Saccharose +	Saccharose −	Saccharose +	Saccharose −
B. communior	*B. communis*	*B. aerogenes*	*B. acidi lactici*

The behavior of the organisms investigated by us is summarized in Table II. We have included a large number of tests that were not required for differentiation purposes because we hoped to find a correlation between these reactions and the production of histamine. Such a relationship seems, however, not to exist. The organisms Coli K, H, B, 88, 51, 80, 84, 90, 52, 74, and Schwartz were isolated from human feces and investigated as soon as they had been obtained in pure culture. The other strains were stock cultures obtained from a variety of sources. Some of these strains have been growing on artificial media for years.

Bacillus coli communior.

Of the organisms investigated, eight belonged to this group. Their behavior on our synthetic medium is summarized in Table III.

Coli P-3-19.—This organism did not rupture the imidazole ring to an appreciable extent. Of the histidine originally introduced, 29.7 per cent was converted into histamine and 59 per cent was recovered unchanged. Imidazoles other than histamine were not formed. Of the ammonia originally introduced, 42 per cent was removed by the microorganisms.

Coli K (*red*).—There is a sufficient discrepancy between the amino nitrogen and color values for both the histidine and the histamine fractions to suggest a slight rupture of the imidazole ring with the production of primary amino groups. Of the histidine originally introduced, 37.6 per cent was converted into histamine and 50 per cent was recovered unchanged. Imidazoles other than histamine were not formed. Of the ammonia originally introduced, 9.7 per cent was removed by the bacilli.

TABLE II.

Name of strain.	Mobility.	Gram stain.	Colony on endo.	Gelatin slab.	Milk (coagulation of).	Dextrose.	Lactose.	Saccharose.	Galactose.	Levulose.	Maltose.	Raffinose.	Mannitol.	Mannose.	Arabinose.	Xylose.	Dulcitol.	Inulin.	Salicin.	Indole.	Voges-Proskauer.	Histamine production.	Classification.
Coli P-3-19	+	−	Red.	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	Bacillus communior.
" K (red)	−	−	"	−	+	+	+	+				+	+				+					+	
" Y	−	−	"	−	+	+	+	+									+					+	
" Jd	−	−	"	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+		−	
" Lac. Aer.	−	−	"	−	+	+	+	+									+					−	
" 88	−	−	"	−	+	+	+	+				+	+				+					−	
" 51	−	−	"	−	+	+	+	+				−	+				+					−	
" H (white)	−	−	White.	−	+	+	+	+									+						
" cystitis	−	−	Red.	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	−	+	Bacillus communis.
" Wk	−	−	"	−	+	+	+	−	+	+	+	−	+	+	−	−	+	−	−	+	−	−	
" Hm	−	−	"	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	−	−	
" Cs	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	+	−	−	+	−	−	
" K (white)	−	−	White.	−	+	+	+	−				−	+				+					−	
" bovis No. 3	−	−	Red.	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	Bacillus aerogenes.
" " " 4	−	−	"	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+		−	
" 80	−	−	"	−	+	+	+	+				+	+				−					−	
" 84	−	−	"	−	+	+	+	+				+	+				−					−	
" 90	−	−	"	−	+	+	+	+				+	+				−					−	

Coli B.	−	−	Red.	−	+	+	+	−				−	+				−					−	Bacillus acidi lactici.
" H.	−	−	"	−	+	+	+	−									−					−	
" P-1-19.	−	−	"	−	+	+	+	−	−	+	+	−	+	+	+	+	−	−	−	+	−	−	
" P-2-19.	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	−	−	−	+	−	−	
" P-4-19.	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	−	
" P-5-19.	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	+	
" P-6-19.	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	−	
" I (K).	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	−	−	−	+	−	−	
" Schwartz.	−	−	"	−	+	+	+	−				+	−				−						
" 52.	−	−	"	−	+	+	+	−				−	+				−					−	
" 51 (white).	−	−	White.	−	+	+	+	−				−	+				−					−	
" 74.	−	−	Red.	−	+	+	+	−				−	+				−					−	

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli P-3-19.	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Match perfect. 96%	0.10 cc. = 5.9 mm. 0.20 " = 11.8 " Match good. 59%	1.18 cc. N_2 at 22° an[d] 745 mm. 0.1327 gm. histidin[e] dichloride. 66.3%
Coli K (red).	0.10 cc. = 10.8 mm. 0.20 " = 21.6 " Match perfect. 108%	0.10 cc. = 5.0 mm. 0.20 " = 10.0 " Match perfect. 50%	1.14 cc. N_2 at 26° an[d] 750 mm. 0.1264 gm. histidin[e] dichloride. 63.2%
Coli Y, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.63 cc. N_2 at 27.5[°] and 744 mm. 0.1795 gm. histidin[e] dichloride. 89.7%
30 days.	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Match perfect. 74%	0.10 cc. = 6.6 mm. 0.20 " = 13.2 " Match good. 66%	2.46 cc. N_2 at 24° an[d] 748 mm. 0.275 gm. histidin[e] dichloride. 137.5%
Coli Jd.	0.10 cc. = 10.1 mm. 0.20 " = 20.2 " Match perfect. 101%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.55 cc. N_2 at 22° an[d] 743 mm. 0.1738 gm. histidin[e] dichloride. 86.9%
Coli Lac. Aer., 7 days.	0.10 cc. = 8.7 mm. 0.20 " = 17.4 " Match perfect. 87%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. 75%	1.37 cc. N_2 at 16° an[d] 750 mm. 0.1597 gm. histidin[e] dichloride. 79.8%
14 days.	0.10 cc. = 5.2 mm. 0.20 " = 10.4 " Match perfect. 52%	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match good. 45%	1.81 cc. N_2 at 23° an[d] 748 mm. 0.2032 gm. histidin[e] dichloride. 101.6%

* Colors matched against the (CR-MO) standard.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	pH	pH
.05 cc. = 7.2 mm. .10 " = 14.4 " olor develops like that of histamine.	0.048 gm. of histamine in entire test solution. 29.7% of histamine present.	0.46 cc. N_2 at 22° and 748 mm. 0.042 gm. histamine dichloride. 26%	21.0 Hence the ≈ of 15 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.9
.05 cc. = 9.1 mm. .10 " = 18.2 " olor develops like that of histamine.	0.0607 gm. of histamine in entire test solution. 37.6% of histamine present.	0.9 cc. N_2 at 30° and 748 mm. 0.0785 gm. histamine dichloride. 48.6%	32.5 Hence the ≈ of 3.5 cc. of 0.1 N NH_3 used by the microorganisms.	7.4	5.4
.50 cc. = 4.8 mm. .00 " = 9.6 " olor develops like that of histamine.	0.00128 gm. of histamine in entire test solution. 1.98%		20.0 Hence the ≈ of 16 cc. of 0.1 N NH_3 used by microorganisms.	7.3	6.0
.20 cc. = 4.0 mm. .40 " = 8.0 " olor develops like that of histamine.	0.00668 gm. of histamine dichloride in entire test solution. 4.1%		None. Hence the ≈ of 36 cc. of 0.1 N NH_3 used by microorganisms.	7.3	4.6
None.			28.0 Hence the ≈ of 8 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.6
None.			36.7 Hence the ≈ of 0.7 cc. of 0.1 N NH_3 was produced by microorganisms.	7.3	5.8
None.			38.0 Hence the ≈ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	5.4

TABLE III-

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli 88.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.3 mm. 0.20 " = 16.6 " Match perfect. 83%	1.62 cc. N_2 at 27° and 746 mm. 0.1775 gm. histidine dichloride 88.8%
Coli 51.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	1.62 cc. N_2 at 27° and 746 mm. 0.1775 gm. histidine dichloride. 88.8%

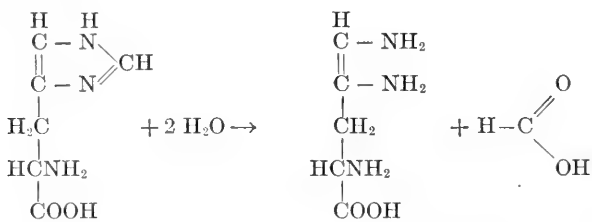
ncluded.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			37.0 Hence the ≈ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.4
None.			33.75 Hence the ≈ of 2.25 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.4	5.6

Coli Y.—During the first 2 weeks of incubation, 8 per cent of the introduced histidine and 44 per cent of the introduced ammonia were removed by the microorganisms. Of the histidine originally introduced, about 2 per cent was converted into histamine. There is no evidence that the imidazole ring was ruptured because the check between the histidine values by amino nitrogen and colorimetric determinations is perfect.

During the next 16 days of incubation an additional 2 per cent of histamine was produced. The liquid was *free from ammonia;* hence 100 per cent of the ammonia originally introduced was removed by the organisms. The most striking observation, however, was the discrepancy between the histidine values obtained by the colorimetric and the amino nitrogen methods.

In a previous paper we called attention to the fact that histidine might be decomposed by microorganisms according to the following structure:



The triamino compound formed, because of its carboxyl group, would form a sodium salt, in the presence of a strong alkali, that should be soluble in water and difficultly soluble in amyl alcohol. In the course of our standard procedure this triamino compound should appear together with histidine in the histidine fraction. Although histidine contains but one primary amino group, the triamino compound contains three such groups; hence each molecule of triamino compound would give three times as much nitrogen, by the Van Slyke method, as a molecule of histidine.

Going back to the table we find that 90 per cent of the introduced histidine was left unchanged at the end of 14 days. After 30 days of incubation, 66 per cent of histidine was present and 2 additional per cent of histamine had been produced. In all, then, 68 of the 90 per cent of histidine left after 14 days of incubation can be accounted for colorimetrically. What became of the 22 per cent of histidine that cannot be accounted for colorimetrically? If we assume that all of this histidine was converted into triamino compound, an amount of N_2 equivalent to 22 times 3 or 66 per cent of histidine would be evolved in an amino nitrogen determination.

If to this we add the nitrogen evolved by the 66 per cent of histidine present in the same liquid, one would expect to obtain an amount of nitrogen equivalent to 132 per cent of histidine which compares very well with the 137.5 per cent actually obtained.

We realize, of course, that an excess of amino nitrogen may not necessarily indicate the presence of a histidine disruption product. It is possible that non-volatile, carboxylated amino compounds, possibly amino-acids, might be synthesized from ammonia and glycerol, and if these were present, the amino nitrogen figure would be high. At present, however, we are inclined to believe that a triamino compound is responsible for the excess amino nitrogen because a quantitative relationship similar to the one given above has been found to hold in five other cases. A quantitative agreement might be obtained once or twice by accident; but it seems hardly reasonable to assume that the accident should occur six times.

The formula of the triamino compound would lead one to believe that the compound might have some physiological activity. A search of the literature revealed the fact that diamino acetylene derivatives have not yet been prepared. We hope to make the preparation and properties of this triamino compound the subject of a subsequent paper.

Finally we can raise the question, *why do certain microorganisms rupture the imidazole ring with the liberation of free amino groups?* Two reasons suggest themselves. This type of nuclear disruption is the most certain way to expose for future use all of the nitrogen and carbon of the molecule. This is also a decomposition that converts a feebly basic substance into one that is strongly basic.

Coli Jd.—This organism did not rupture the imidazole ring. Of the histidine originally introduced, 86 per cent was recovered unchanged. Histamine and other imidazoles were not formed. Of the ammonia originally introduced 22 per cent was removed by the microorganisms.

Coli Lac. Aer.—During the first 7 days of incubation, this rapidly growing organism reduced the histidine concentration of the solution to 75 per cent of its initial value. There is practically no indication that a nuclear rupture occurred. The concentration of ammonia in the solution was *greater* after 7 days of incubation than it was at the outset of the experiment. Some of this ammonia must have been derived either from the disrupted histidine, or from the potassium nitrate.

The second 7 day period of incubation reduced the histidine concentration to 45 per cent of its initial value. Some of this histidine was apparently converted into triamino compound and some into ammonia. Although there is no doubt, in this case, that a carboxylated amino compound was formed from the histidine, quantitative proof for the formation of a triamino compound is lacking because the decomposition continued beyond this stage with the production of ammonia. Histamine and other imidazoles were not formed.

The increase in the ammonia figure might suggest that this organism was unable to utilize ammonia as a source of nitrogen, and that the imidazole ring was ruptured to render nitrogen available. This was, however, not the case because this organism grows splendidly on a medium that contains only inorganic salts, glycerol, and NH_4Cl. On this histidine-free medium the ammonia value drops from 36 to 25 cc. of 0.1 N HCl in the course of 2 weeks. The rupture of the imidazole ring might, therefore, have been resorted to in an attempt to lower the hydrogen ion concentration of the cell protoplasm.

Coli 88.—This organism did not rupture the imidazole ring with the production of appreciable quantities of a carboxylated amino compound; but some of the histidine nitrogen must have been converted into ammonia because the concentration of ammonia at the end of the 14 day incubation period was greater than the initial concentration. Of the histidine originally introduced, 83 per cent was recovered unchanged. Histamine and other imidazoles were not formed.

Coli 51.—The results obtained with this organism were so nearly like those obtained with Coli 88 that a detailed discussion seems superfluous. In this case, however, the ammonia consumption was more rapid than its production so that the initial ammonia value was reduced by 6.2 per cent.

Bacillus coli communis.

Of the organisms investigated, five belong to this group. Their behavior on our synthetic medium is summarized in Table IV.

Coli cystitis.—This is the organism that was used in our earlier work.[1] Since its behavior has been described in detail in our previous articles, it seems unnecessary to do more here than to call attention to the fact that it has lost none of its power of decarboxylation. In this experiment 57 per cent of the histidine originally introduced was converted into histamine as compared to a 50 per cent conversion obtained by us some 2 years ago.

Coli Wk.—This organism ruptured the imidazole ring to a considerable extent, apparently with the formation of a carboxylated amino compound. Quantitative proof for the formation of a triamino compound is, however, lacking in this case. Of the histidine originally introduced 76 per cent was recovered. Histamine and other imidazoles were not formed. Of the

ammonia originally introduced, 8.3 per cent was removed by the microorganisms.

Coli Hm.—This organism seems not to have attacked the histidine at all because 96 per cent of this amino-acid was recovered. The nitrogen requirements were obviously supplied by the ammonia whose final concentration was only 75 per cent of that originally introduced.

Coli Cs.—This organism did not rupture the imidazole ring with the formation of a carboxylated amino compound. Of the histidine originally introduced, 86 per cent was recovered. Histamine or other imidazoles were not formed. Of the ammonia originally introduced, 25 per cent was removed by the microorganisms.

Coli K (White).—This organism ruptured the imidazole ring to the extent of 8 per cent, apparently with the formation of a triamino compound (see under Coli Y). Of the histidine originally introduced, 92 per cent was recovered. If we assume that the 8 per cent of histidine that disappeared in the course of 2 weeks incubation, was quantitatively converted into triamino compound, the total amino nitrogen value of the liquid should have been eight times 3 plus 92 equals 116 per cent, which compares very well with the 113.4 per cent actually obtained. Histamine and other imidazoles were not formed. A small amount of ammonia was produced either from the histidine or from the potassium nitrate.

Bacillus lactis aerogenes.

Of the organisms investigated, five belong to this group. Their behavior on our synthetic medium is summarized in Table V.

Coli bovis 3.—During the first 2 weeks of incubation, this organism reduced the histidine concentration of the solution to 92 per cent of its initial value and removed 17 per cent of the ammonia that was originally introduced. There is no indication that a nuclear rupture occurred. Histamine and other imidazoles were not produced.

After 30 days of incubation, the histidine concentration was reduced to 84 per cent of its initial value. During this period, 2.4 per cent of histidine was converted into histamine. In all, then, 86.4 per cent of histidine can be accounted for colorimetrically, after 30 days of incubation. After 14 days of incubation, 92 per cent of histidine was recovered. If we assume that the 5.6 per cent of histidine that disappeared during the second 16 day period was converted quantitatively into triamino compound, an amino nitrogen value of 5.6 times 3 plus 84 equals 100.8 per cent should have been obtained for the histidine fraction which compares very well with the 103.5 per cent actually obtained. This organism seems, therefore, to have converted 5.6 per cent of histidine into triamino compound. Of the ammonia originally introduced, 28.5 per cent was removed by the microorganisms.

TABLE IV—*Bacill*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) wit 5 cc. of test solution.
Coli cystitis.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Color developed very rapidly. 112%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. 40%	0.85 cc. N_2 at 20° an 750 mm. 0.097 gm. histidi dichloride. 48.5%
Coli Wk.	0.10 cc. = 8.4 mm. 0.20 " = 16.9 " Match perfect. 84%	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match good. 76%	1.95 cc. N_2 at 22° an 748 mm. 0.220 gm. histidin dichloride. 110%
Coli Hm.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. 103%	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Match perfect. 96%	1.80 cc. N_2 at 20° an 750 mm. 0.206 gm. histidin dichloride. 103%
Coli Cs.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.57 cc. N_2 at 23° an 750 mm. 0.1768 gm. histidin dichloride. 88.4%
Coli K (white).	0.10 cc. ≐ 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.2 mm. 0.20 " = 18.3 " Match perfect. 92%	2.07 cc. N_2 at 28° an 750 mm. 0.2268 gm. histidin dichloride. 113.4%

* Colors matched against the (CR–MO) standard.

i communis.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
05 cc. = 13.8 mm. 10 " = 27.5 " olor develops like that of histamine.	0.092 gm. of histamine dichloride in the entire test solution. 57% of histamine present.	1.04 cc. N_2 at 20° and 754 mm. 0.0964 gm. histamine dichloride. 59.8%	27 Hence the ≈ of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.4
None.			33 Hence the ≈ of 3 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	6.4
None.			27 Hence the ≈ of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.2
None.			27 Hence the ≈ of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.8
None.			37 Hence the ≈ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.5

TABLE V—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli bovis 3, 14 days.	0.10 cc. = 10.6 mm. 0.20 " = 21.2 " Match perfect. 106%	0.10 cc. = 9.2 mm. 0.20 " = 18.4 " Match perfect. 92%	1.65 cc. N_2 at 27° and 749 mm. 0.182 gm. histidine dichloride. 91%
30 days.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.4 mm. 0.20 " = 16.7 " Match perfect. 84%	1.85 cc. N_2 at 21° and 738 mm. 0.207 gm. histidine dichloride. 103.5%
Coli bovis 4.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. 103%	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	1.93 cc. N_2 at 26° and 745 mm. 0.212 gm. histidine dichloride. 106%
Coli 80.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.97 cc. N_2 at 30° and 747 mm. 0.213 gm. histidine dichloride. 106.5%
Coli 84.	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.79 cc. N_2 at 25° and 751 mm. 0.2000 gm. histidine dichloride. 100%
Coli 90.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.51 cc. N_2 at 21° and 752 mm. 0.172 gm. histidine dichloride. 86%

* Colors matched against the (CR–MO) standard.

lactis aerogenes.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			29.7 Hence the ≎ of 6.3 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.4
0.40 cc. = 4.7 mm. 0.80 " = 8.4 " Color develops like that of histamine.	0.0039 gm. of histamine dichloride in the entire test solution. 2.4% of histamine present.		25.7 Hence the ≎ of 10.3 cc. of 0.10 N NH_3 used by the microorganisms.	7.3	5.2
None.			36.0 Hence appreciable quantities of NH_3 seem not to have been removed by the microorganisms.	7.4	5.4
None.			29 Hence the ≎ of 7 cc. of 0.1 N NH_3 used by the microorganisms.	7.4	5.4
None.			37 Hence the ≎ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			37.5 Hence the ≎ of 1.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6

Coli bovis 4.—This organism did not grow as well as the other coli and it seems not to have attacked appreciable quantities of either the histidine or the ammonia.

Coli 80.—This colon bacillus ruptured the imidazole ring with the formation of carboxylated amino compounds. Of the histidine originally introduced, 80 per cent was recovered. Histamine and other imidazoles were not formed. The ammonia concentration was reduced by 19.5 per cent.

Coli 84.—A nuclear rupture with the formation of carboxylated amino compounds is again indicated in this case. Of the histidine originally introduced, 86 per cent was recovered. Some ammonia was produced either from the disrupted histidine or from the potassium nitrate. Histamine or other imidazoles were not formed.

Coli 90.—The liquid finally obtained in this case was free from carboxylated amino compounds other than histidine. Of this amino-acid, 84 per cent was recovered unchanged. Some ammonia was produced either from the disrupted histidine or from the potassium nitrate. Histamine or other imidazoles were not formed.

Bacillus acidi lactici.

Of the organisms investigated, twelve belong to this group. Their behavior on our synthetic medium is summarized in Table VI.

Ten of these organisms gave results that are qualitatively identical; so they can be discussed collectively. Only Coli P-2–19 and P–5–19 require special consideration.

All of the ten similar organisms ruptured the imidazole ring with the production of carboxylated amino compounds. The disruption did not proceed quantitatively to the formation of triamino compound in any of these cases. Histamine and other imidazoles were not formed. Some of the organisms—Coli H, Schwartz, 52, 51 (white), and 74—produced ammonia, and others —Coli P–1–19, P–4–19, P–6–19, and I (K)—removed some from the solution.

Coli P-2-19.—The formation of a carboxylated triamino compound is again quantitatively indicated in this case. Of the histidine originally introduced, 95 per cent was recovered. If we assume that the 5 per cent of histidine that disappeared was quantitatively converted into a triamino compound, an amino nitrogen value of five times 3 plus 95 or 110 per cent should have been obtained which agrees almost exactly with the 110.2 per cent actually obtained. Histamine and other imidazoles were not formed. Of the ammonia originally introduced, 57 per cent was removed by the microorganisms.

Coli P-5-19.—This interesting organism converted 14.5 per cent of the histidine originally introduced, into histamine. Since 79 per cent of histidine was recovered unchanged, 93.5 per cent of the original histidine can be accounted for colorimetrically. The amino nitrogen determination on the histidine fraction gave a value of 100.5 per cent calculated as histidine. Of this amino nitrogen only 79 per cent could have been derived from histidine. If we assume that the remaining 21.5 per cent of amino nitrogen was derived from the triamino compound, this would account for 7.17 per cent of histidine; *i.e.*, 21.5 divided by 3. Summing up, then, we have

79.0	per cent as histidine.
14.5	" " " histamine.
7.17	" " " triamino compound.
100.67	" " total recovery.

which is a truly remarkable agreement.

PART II.

The Products Formed from Histidine by the Action of Other Members of the Colon Typhoid Group.

The organisms investigated were *Bacillus enteritidis, Bacillus typhosus, Bacillus paratyphosus* A (3 strains), *Bacillus dysenteriæ* Flexner, *Bacillus dysenteriæ* Morgan, *Bacillus dysenteriæ* Shiga, *Bacillus fæcalis alcaligenes* I, and *Bacillus fæcalis alcaligenes III*. The behavior of these organisms on our synthetic medium is summarized in Table VII.

B. enteritidis 228.—This organism grew very well on the liquid medium. There is no evidence of a rupture of the imidazole ring after 7 days of incubation although 20 per cent of the introduced histidine disappeared during this time interval. Of the ammonia originally introduced 32 per cent was removed by the microorganisms. Histamine and other imidazoles were not formed.

The attack on the remaining histidine was so intense, during the second 7 day period, that 40 per cent of that amino-acid disappeared. A nuclear rupture occurred with the formation of some carboxylated amino compound and considerable ammonia. Histamine and other imidazoles were not formed.

B. typhosus.—This organism grew poorly on our medium. Histamine and other imidazoles were not formed. Very little acid was produced. Of the ammonia originally introduced, 80 per cent was recovered. The results on the histidine fraction suggest that a small amount of histidine was deaminized because the colorimetric value for histidine ran higher than the amino nitrogen value. The conversion was too slight, however, to be of particular significance.

TABLE VI—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli B.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.2 mm. 0.20 " = 18.4 " Match perfect. 92%	1.90 cc. N_2 at 28° and 747 mm. 0.208 gm. histidine dichloride. 104%
Coli H.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.80 cc. N_2 at 30° and 747 mm. 0.1945 gm. histidine dichloride. 97.2%
Coli P-1-19.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.1 mm. 0.20 " = 18.2 " Match perfect. 91%	1.70 cc. N_2 at 21° and 744 mm. 0.192 gm. histidine dichloride. 96%
Coli P-2-19.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	1.99 cc. N_2 at 25° and 745 mm. 0.2204 gm. histidine dichloride. 110.2%
Coli P-4-19.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.4 mm. 0.20 " = 16.7 " Match perfect. 84%	1.85 cc. N_2 at 22° and 747 mm. 0.209 gm. histidine dichloride. 104.5%
Coli P-5-19.	0.10 cc. = 9.8 mm. 0.20 " = 19.4 " Match perfect. 98%	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. 79%	1.77 cc. N_2 at 20° and 745 mm. 0.201 gm. histidine dichloride. 100.5%
Coli P-6-19.	0.10 cc. = 9.4 mm. 0.20 " = 18.9 " Match perfect. 94%	0.10 cc. = 9.4 mm. 0.20 " = 18.8 " Match perfect. 94%	1.81 cc. N_2 at 21° and 746 mm. 0.205 gm. histidine dichloride. 102.5%

* Colors matched against the (CR-MO) standard.

cidi lactici.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.				7.4	5.8
None.			38 Hence the ≈ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			31 Hence the ≈ of 5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.7
None.			15.5 Hence the ≈ of 20.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.7
None.			32.5 Hence the ≈ of 3.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.9
.10 cc. = 7.0 mm. .20 " = 14.0 " Color develops like that of histamine.	0.0235 of histamine dichloride in the entire test solution. 14.5% of histamine present.	0.35 cc. N_2 at 22° and 744 mm. 0.0317 gm. histamine dichloride. 19.6%	35.5 Hence the ≈ of 0.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	6.2
None.			27 Hence the ≈ of 9 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	6.0

TABLE VI

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli 1 (K).	0.10 cc. = 9.0 mm. 0.20 " = 18.1 " Match perfect. 90%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.71 cc. N_2 at 22.5 and 745 mm. 0.1925 gm. histidine dichloride. 96.2%
Coli Schwartz.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. 89%	1.89 cc. N_2 at 28° and 749 mm. 0.207 gm. histidine dichloride. 103.5%
Coli 52.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.87 cc. N_2 at 27° and 746 mm. 0.205 gm. histidine dichloride. 102.5%
Coli 51 (white).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	1.89 cc. N_2 at 28° and 749 mm. 0.207 gm. histidine dichloride. 103.5%
Coli 74.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.8 mm. 0.20 " = 17.6 " Match perfect. 88%	1.83 cc. N_2 at 28° and 747 mm. 0.200 gm. histidine dichloride. 100%

Concluded.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			32.5 Hence the ≈ of 3.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.3	5.5
None.			38.5 Hence the ≈ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			39 Hence the ≈ of 3 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.4
None.			38 Hence the ≈ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	7.0
None.			37 Hence the ≈ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.5

TABLE VII—*Col*

Name of strain.	Total color value of test solution of histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) wit 5 cc. of test solution.
Bacillus enteritidis 228, 7 days.	0.10 cc. = 8.6 mm. 0.20 " = 17.1 " Match perfect. 86%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.46 cc. N_2 at 29° an 746 mm. 0.1584 gm. histidir dichloride. 79.2%
14 days.	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match poor. Color too yellow. 40%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	1.25 cc. N_2 at 23° an 745 mm. 0.140 gm. histidir dichloride. 70%
Bacillus typhosus.	0.10 cc. = 9.6 mm. 0.20 " = 19.1 " Match perfect. 96%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.35 cc. N_2 at 21° an 743 mm. 0.152 gm. histidir dichloride. 76%
Bacillus paratyphosus A 3.	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	0.10 cc. = 8.3 mm. 0.20 " = 16.7 " Match perfect. 83%	1.47 cc. N_2 at 20° an 738 mm. 0.1655 gm. histidine dichloride. 82.7%
Bacillus paratyphosus A 4, 7 days.	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	1.73 cc. N_2 at 28° an 746 mm. 0.1886 gm. histidir dichloride. 94.3%
14 days.	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	1.95 cc. N_2 at 24° an 753 mm. 0.2195 gm. histidin dichloride. 109.7%
Bacillus paratyphosus A (K).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine. 100%	0.95 cc. N_2 at 24° an 753 mm. 0.107 gm. histidin dichloride. 53.5%

* Colors matched against the (CR–MO) standard.

yphoid Group.

istidine converted into imidazole ropionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	24.5 Hence the ≎ of 11.5 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	6.0
None.	None.	None.	39.5 Hence the ≎ of 15 cc. of 0.1 N NH_3 was produced during the second 7 day period.	7.3	5.2
	None.	None.	29 Hence the ≎ of 7 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	6.6
None.	None.	None.	31.5 Hence the ≎ of 4.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
None.	None.	None.	39 Hence the ≎ of 3 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	6.4
None.	None.	None.	38 Hence the ≎ of 1 cc. of 0.1 N NH_3 was removed during the second 7 day period.	7.3	5.4
0.034 gm. 27.7%	None.	None.	30.5 Hence the ≎ of 5.5 cc. of 0.10 N NH_3 was removed by the microorganisms.	7.3	6.8

TABLE VI

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) wit 5 cc. of test solution.
Bacillus dysenteriæ Flexner.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine. 100%	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Color too pink and develops too rapidly for histamine. 96%	0.91 cc. N_2 at 22° an 745 mm. 0.1025 gm. histidi dichloride. 51.2%
Bacillus dysenteriæ Morgan.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect, color develops rapidly. 95%	0.10 cc. = 7.8 mm. 0.20 " = 15.6 " Match perfect, color develops rapidly. 78%	1.15 cc. N_2 at 22° an 743 mm. 0.129 gm. histidi dichloride. 65%
Bacillus dysenteriæ Shiga.	0.10 cc. = 11.3 mm. 0.20 " = 22.6 " Color too pink and develops too rapidly for histamine. 113%	0.10 cc. = 9.3 mm. 0.20 " = 18.5 " Color too pink and develops too rapidly for histamine. 93%	1.24 cc. N_2 at 19° an 748 mm. 0.1423 gm. histidine dichloride. 71.1%
Bacillus fæcalis alcaligenes I.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Color slightly too pink. 97%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Color slightly too pink for histidine. 70%	0.80 cc. N_2 at 23° a 749 mm. 0.090 gm. histidi dichloride. 45%
Bacillus fæcalis alcaligenes III, 3 days.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match good. 90%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Match perfect. 70%	1.30 cc. N_2 at 23° a 750 mm. 0.1464 gm. histidi dichloride. 73.2%
14 days.	0.10 cc. = 9.2 mm. 0.20 " = 18.5 " Match perfect. 92%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Match perfect. 70%	1.17 cc. N_2 at 23° a 749 mm. 0.1317 gm. histidi dichloride. 66%

Concluded.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
0.033 gm. 27.1%	None.	None.	34.5 Hence the $\approx$ of 1.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.010 gm. 8.1%	None.	None.	35.5 Hence the $\approx$ of 0.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.016 gm. 13%	None.	None.	33 Hence the $\approx$ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.018 gm. 14.65%	None.	None.	32.5 Hence the $\approx$ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4
None.	None.	None.	32.5 Hence the $\approx$ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4
None.	None.	None.	33 Hence the $\approx$ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.6

B. paratyphosus A 3.—This organism grew very poorly on our medium, apparently because an available carbon source was lacking. Both the glycerol and the histidine remained largely unattacked. Histamine, other imidazoles, and acid were not formed. Of the ammonia originally introduced, 13 per cent was removed by the bacilli.

B. paratyphosus A 4.—This organism grew far better than *B. paratyphosus* A 3. Some of the glycerol was metabolized with the production of acid. The imidazole ring was ruptured to a considerable extent with the formation of carboxylated amino compounds and ammonia. Histamine and other imidazoles were not formed.

B. paratyphosus A (K).—This organism deaminized histidine with the formation of imidazole acetic, propionic, lactic, or acrylic acids because the color produced by the histidine fraction was far too red for histidine and the amino nitrogen value was much lower than the colorimetric value. If we assume that *the amino nitrogen was derived exclusively from histidine*, the presence of a maximum of 53.5 per cent of that substance is indicated. Colorimetrically this would give a reading of 5.35 mm. (CR − MO) for 0.10 cc. of the diluted test solution. The reading obtained was 10.0 mm. (CR − MO) for 0.10 cc. The difference between these two readings—4.65 mm. (CR − MO) or 5.12 mm. (CR)[8]—must have been due to either imidazole acetic, propionic, lactic, or acrylic acids. Which of these acids was present was not determined; but the excess color value was calculated as imidazole propionic acid using the table that was previously published for that substance. The calculations show that 0.034 gm. of imidazole propionic acid was present; hence 27.7 per cent of the histidine originally introduced was deaminized.

This organism grew poorly, used glycerol to only a small extent, produced very little acid, and removed the equivalent of 16 per cent of the ammonia originally introduced. Histamine was not formed.

B. dysenteriæ Flexner, Morgan, and Shiga.—The three classes of dysentery bacilli can most advantageously be discussed together because, although the results are quantitatively somewhat different, they are qualitatively identical. In every case the color obtained with the histidine fraction was too red to have been due only to histidine. The colorimetric values were higher than the amino nitrogen values on this fraction; hence histidine was not the only imidazole present. When the discrepancies between the values obtained colorimetrically and by the amino nitrogen method are calculated as imidazole propionic acid, the presence of 0.033 gm. (27.1 per cent), 0.01 gm. (8.1 per cent), and 0.016 gm. (13 per cent) of this substance is indicated for Flexner, Morgan, and Shiga respectively.

These organisms grew poorly, used glycerol only to a small extent, produced practically no acid, and removed very little ammonia from the solution. Histamine was not formed.

[8] The (CR) standard is $\frac{10}{11}$ as intense as the (CR − MO) standard.

B. fæcalis alcaligenes I.—The color obtained with the histidine fraction was too red for histidine and the amino nitrogen value was 25 per cent lower than the colorimetric value. This suggests the presence of imidazole acetic, propionic, lactic, or acrylic acids. When the discrepancy between the values obtained colorimetrically and by the amino nitrogen method are calculated as imidazole propionic acid, the presence of 0.018 gm. (14.65 per cent) of this substance is indicated.

This organism grew so poorly that the medium was almost clear after 14 days of incubation. Some of the bacilli were, nevertheless, alive at the end of this period. Little or no use was made of the glycerol, and acid was not produced. Of the ammonia originally introduced, 90 per cent was recovered. Histamine was not formed.

B. fæcalis alcaligenes III.—This organism grew about as poorly as *B. fæcalis alcaligenes* I. During the first 3 days, 30 per cent of the histidine was removed. After that there was practically no change in the histidine content of the liquid. Imidazole propionic acid and histamine were not formed. Of the ammonia originally introduced, 90 per cent was recovered. The slight production of alkalinity during the las 11 days of incubation may have been caused by autolytic changes because apparently the active life of the organisms came to an end after 3 days of meager growth.

PART III.

On the Products Formed from Histidine by the Action of Organisms Other than Those Belonging to the Colon Typhoid Groups.

The organisms investigated were *Bacillus mucosus capsulatus* (2 strains), *Bacillus bifidus, Bacillus influenzæ, Bacillus proteus vulgaris* (2 strains), *Bacillus cloacæ, Streptococcus hæmolyticus,* (2 strains), Pneumococcus Types I, II, III, and IV, and *Bacillus tuberculosis* (5 strains). The behavior of these organisms on our synthetic medium is summarized in Table VIII.

B. mucosus capsulatus.—An excellent growth was obtained. This organism attacked the histidine immediately and so effectively that 75 per cent of it was destroyed in 14 days. The histidine seems to have been attacked because of its nitrogen. The following facts led us to this conclusion:

1. The ammonia concentration remained practically unchanged throughout the entire course of the experiment in spite of the fact that an unusually abundant growth was obtained. The nitrogen requirements of the organism must, therefore, have been obtained from the histidine.

2. Although the histidine was largely removed from the solution, none of its nitrogen appeared as ammonia and only a very small amount of it appeared in the amino condition. During the first few days, all of the nitrogen derived from the disrupted histidine was consumed. Toward the

TABLE VIII—*Miscel*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus mucosus capsulatus L. S., 3 days.	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	0.10 cc. = 7.3 mm. 0.20 " = 14.5 " Match perfect. 73%	1.33 cc. N_2 at 18° and 750 mm. 0.1535 gm. histidine dichloride. 76.8%
14 days.	0.10 cc. = 2.8 mm. 0.20 " = 5.6 " Match fair. 28%	0.20 cc. = 5.1 mm. 0.40 " = 10.2 " Match good. 25.5%	0.75 cc. N_2 at 25° and 745 mm. 0.083 gm. histidine dichloride. 41.5%
Bacillus mucosus capsulatus L. S. plus 0.10 gm. leucine, 14 days.	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match good. 80%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match good. 75%	1.46 cc. N_2 at 21° and 742 mm. 0.1655 gm. histidine dichloride. 82.7%
Bacillus mucosus capsulatus 27 (S), 3 days.	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. 75%	0.46 cc. N_2 at 23° and 752 mm. 0.052 gm. histidine dichloride. 26%
5 days.	0.20 cc. = 5.8 mm. 0.40 " = 11.6 " Match good. 29%	0.20 cc. = 4.9 mm. 0.40 " = 9.8 " Match good. 23%	0.80 cc. N_2 at 21° and 737 mm. 0.0894 gm. histidine dichloride. 44.7%
8 days.	0.50 cc. = 2.4 mm. Color brownish yellow. Match poor. 4.8%	0.50 cc. = 2.0 mm. 1.00 " = 4.0 " Match good. 4.0%	0.75 cc. N_2 at 21° and 737 mm. 0.0838 gm. histidine dichloride. 41.9%
Bacillus bifidus, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.7 mm. 0.20 " = 17.4 " Match perfect. 87%	1.55 cc. N_2 at 23° and 744 mm. 0.173 gm. histidine dichloride. 86.5%

*Colors matched against the (CR–MO) standard.

laneus Organisms.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	36.7 Hence the ≏ of 0.7 cc. of 0.1 N NH_3 was produced.	7.3	6.2
None.	None.	None.	36.7 An ammonia equilibrium was established after 7 days of growth.	7.3	6.8
None.	None.	None.	34 Hence the ≏ of 2 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.4
0.0365 gm. 29.7%	None.	None.	38.5 Hence the ≏ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.2
None.	None.	None.	44 Hence the ≏ of 8 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.1
None.	None.	None.	49 Hence the ≏ of 13 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.0
None.	None.	None.	32.5 Hence the ≏ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus influenzæ, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.55 cc. N_2 at 32° and 746 mm. 0.165 gm. histidine dichloride. 82.5%
Bacillus proteus vulgaris 186, 7 days.	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match good. 76%	0.10 cc. = 6.9 mm. 0.20 " = 13.7 " Match good. 69%	1.13 cc. N_2 at 21° and 754 mm. 0.1294 gm. histidine dichloride. 64.7%
14 days.	0.20 cc. = 9.0 mm. 0.30 " = 13.7 " Color too yellow for histidine. 45%	0.20 cc. = 9.0 mm. 0.30 " = 13.5 " Match fair. Color slightly too yellow. 45%	0.78 cc. N_2 at 21° and 752 mm. 0.089 gm. histidine dichloride. 44.5%
Bacillus proteus vulgaris A. I. K., 14 days.	0.10 cc. = 8.1 mm. 0.20 " = 16.2 " Match perfect. 81%	0.10 cc. = 7.4 mm. 0.20 " = 14.7 " Match perfect. 74%	2.20 cc. N_2 at 20° and 738 mm. 0.2475 gm. histidine dichloride. 123.7%
Bacillus cloacæ I, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	1.85 cc. N_2 at 26° and 745 mm. 0.2036 gm. histidine dichloride. 101.8%
Streptococcus hæmolyticus 4A, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.51 cc. N_2 at 22° and 748 mm. 0.1705 gm. histidine dichloride. 85.2%
Streptococcus hæmolyticus II R, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.65 cc. N_2 at 28° and 746 mm. 0.180 gm. histidine dichloride. 90%

Continued.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
	None.	None.	31.5 Hence the ≏ of 4.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.3
None.	None.	None.	35 Hence the ≏ of 1 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
None.	None.	None.	40 Hence the ≏ of 4 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.1
None.	None.	None.	28 Hence the ≏ of 8 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.2
None.	None.	None.	30 Hence the ≏ of 6 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.5
None.	None.	None.	32.5 Hence the ≏ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.3
None.	None.	None.	33 Hence the ≏ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Pneumococcus Type I, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.48 cc. N_2 at 21° and 750 mm. 0.1684 gm. histidine dichloride. 84.2%
Pneumococcus Type I plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.54 cc. N_2 at 29° and 746 mm. 0.167 gm. histidine dichloride. 84%
Pneumococcus Type II, 14 days.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	0.10 cc. = 7.8 mm. 0.20 " = 15.5 " Match perfect. 78%	1.38 cc. N_2 at 32° and 748 mm. 0.1475 gm. histidine dichloride. 74%
Pneumococcus Type II plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. 89%	2.05 cc. N_2 at 32° and 746 mm. 0.2185 gm. histidine dichloride. 109.2%
Penumococcus Type III, 14 days.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. 79%	1.65 cc. N_2 at 32° and 748 mm. 0.1765 gm. histidine dichloride. 88.2%
Pneumococcus Type III plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.81 cc. N_2 at 32° and 748 mm. 0.1935 gm. histidine dichloride. 96.8%
Pneumococcus Type IV, 14 days.	0.10 cc. = 9.7 mm. 0.20 " = 19.3 " Match perfect. 97%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.47 cc. N_2 at 31° and 748 mm. 0.158 gm. histidine dichloride. 79%

Continued.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	33 Hence the ≈ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
None.	None.	None.	33 Hence the ≈ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	33 Hence the ≈ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the ≈ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the ≈ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the ≈ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
None.	None.	None.	33 Hence the ≈ of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Pneumococcus Type IV plus 0.10 gm. leucine, 14 days.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Match perfect. 97%	0.10 cc. = 7.9 mm. 0.20 " = 16.0 " Match perfect. 79%	1.60 cc. N_2 at 31° and 748 mm. 0.172 gm. histidine dichloride. 86%
Bacillus tuberculosis H. Sp., 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Color too red for histidine. 90%	1.23 cc. N_2 at 22° and 756 mm. 0.140 gm. histidine dichloride. 70%
Bacillus tuberculosis 1305, 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Color too red for histidine. 95%	0.95 cc. N_2 at 20° and 750 mm. 0.1085 gm. histidine dichloride. 54.2%
Bacillus tuberculosis 3161, 45 days.	0.10 cc. = 9.6 mm. 0.20 " = 19.3 " Color too red for histidine. 96%	0.10 cc. = 8.1 mm. 0.20 " = 16.2 " Color too red for histidine. 81%	0.95 cc. N_2 at 20° and 752 mm. 0.109 gm. histidine dichloride. 54.5%
Bacillus tuberculosis O. H., 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Color too red for histidine. 95%	1.09 cc. N_2 at 21° and 761 mm. 0.126 gm. histidine dichloride. 63%
Bacillus tuberculosis (Novy) K_1, 5 days.	0.10 cc. = 5.8 mm. 0.20 " = 11.5 " Match fair. Color too yellow. 58%	0.10 cc. = 4.7 mm. 0.20 " = 9.2 " Match fair. Color too yellow. 47%	1.27 cc. N_2 at 18° and 740 mm. 0.1446 gm. histidine dichloride. 72.3%
45 days.	1.00 cc. = 15.0 mm. Color brown. Match poor. 15%	1.00 cc. = 12.0 mm. Color brown. Match poor. 12%	0.81 cc. N_2 at 21° and 762 mm. 0.0936 gm. histidine dichloride. 46.8%

Concluded.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	32 Hence the ≎ of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.015 gm. 12.2%	None.	None.	27 Hence the ≎ of 9 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.030 gm. 24.5%	None.	None.	28.5 Hence the ≎ of 7.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.020 gm. 16.3%	None.	None.	31 Hence the ≎ of 5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
0.023 gm. 18.7%	None.	None.	32.5 Hence the ≎ of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	6.9
None.	None.	None.	38.5 Hence the ≎ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.2
None.	None.	None.	37.5 Hence the ≎ of 1 cc. of 0.1 N NH_3 was removed during the 40 day period.	7.3	6.8

end of the 2 week period non-volatile amino nitrogen was liberated slightly faster than it was being consumed.

3. When leucine was added to the medium, along with the histidine, the growth of the organisms was augmented; but it was the leucine nitrogen that was removed from the solution, the histidine being largely unattacked. That the leucine nitrogen was removed is proved by the fact that the amino nitrogen value on the histidine fraction ran only slightly higher than the colorimetric value for histidine.

One might be led to assume that this organism cannot use ammonia to satisfy its nitrogen requirements. This is, however, not the case because a very good growth is obtained in a medium containing only glycerol and ammonium chloride together with the usual inorganic salts. In this case considerable ammonia is removed by the microorganisms. It seems that this organism used amino nitrogen in preference to ammonia nitrogen. Histamine and other imidazoles were not formed.

In addition to the above stock culture, two strains of *Bacillus mucosus capsulatus* were isolated in this laboratory, from the feces of two cases of colitis. They were quantitatively almost identical in their action. The horizontal Columns 4, 5, and 6 contain a summary of the behavior of one of them.

An excellent growth was obtained. The amino nitrogen value of the histidine fraction, obtained after 3 days of incubation, shows that a maximum of only 26 per cent of unchanged histidine could have been present. The color obtained with this fraction was too red to have been exclusively due to histidine, and the color value was far too high to agree with the amino nitrogen value; hence the presence of imidazole acetic, propionic, lactic, or acrylic acids is indicated. This discrepancy between the color value and the amino nitrogen value is equal to 0.0365 gm. (29.7 per cent) calculated as imidazole propionic acid. This organism therefore attacks the histidine in two distinct ways to obtain nitrogen. Some of the molecules are deaminized, others suffer a nuclear disruption. Some, but not much, ammonia is generated during the first 3 days.

During the next 2 days the imidazole propionic (?) acid formed at first was destroyed with the liberation of some non-volatile primary amine and considerable ammonia. The color obtained with the histidine fraction in this case was just like that obtained with histidine. We have assumed, therefore, that the color was at least *largely* due to histidine. If this conclusion is correct, the attack during this 2 day period was directed almost exclusively against the imidazole propionic acid formed during the first 3 days.

During the next 2 days the remaining histidine was destroyed almost completely with the liberation of ammonia. Histamine was not formed at any time.

The three strains of *Bacillus mucosus capsulatus* studied by us satisfied their nitrogen requirements at the expense of the amino-acid histidine in preference to ammonia. Two of the strains deaminized histidine and ruptured the nucleus simultaneously. The other strain merely ruptured the nucleus. Glycerol was, in every case, the chief source of carbon.

B. bifidus.—This organism grew very poorly on our medium, apparently because an available carbon source was lacking. The glycerol originally introduced seemed not to have been attacked. Of the histidine originally introduced, 87 per cent was recovered. There is no evidence that the nucleus was ruptured. The equivalent of 90 per cent of the initial ammonia was recovered. Histamine was not formed. When leucine was added to the medium the organisms grew for a few days during which time *all* the leucine nitrogen disappeared and some acid was produced. Histamine was not produced in this case.

B. influenzæ.—This organism grew very poorly on our medium. There is slight evidence for the formation of imidazole propionic (etc.) acids. Of the ammonia originally introduced, 91 per cent was recovered. Acid was not produced; glycerol was not utilized.

When leucine (0.10 gm.) was added to the medium, the organisms grew for a few days. They apparently found, in leucine, an easily available source of carbon and nitrogen because none of the leucine nitrogen could be accounted for at the end of 14 days. In this case 95 per cent of the histidine originally introduced was recovered. Histamine was not formed in any case.

B. proteus vulgaris 186.—This organism grew poorly on our medium apparently because an easily available source of carbon was lacking. Acid was not produced; glycerol was not attacked. The histidine was progressively destroyed, the excess nitrogen appearing as ammonia. The organisms must, therefore, have destroyed the histidine to obtain carbon. Histamine and other imidazoles were not formed.

B. proteus vulgaris A. I. K.—This organism grew very well on our medium. Glycerol was consumed and acids were produced. The great discrepancy between the color and amino nitrogen values for histidine is good evidence that a carboxylated amino compound was produced from the histidine. Since this organism was able to use ammonia as a source of nitrogen, the excessive amine production could hardly have been resorted to because of the nitrogen thus rendered available. The amines were more probably produced to lower the hydrogen ion concentration of the cell protoplasm. The equivalent of 77 per cent of the ammonia originally introduced was recovered. Histamine and other imidazoles were not formed.

B. cloacæ I.—As far as we could tell, this organism died shortly after it was introduced into our medium. The histidine and the glycerol were not attacked.

Streptococcus hæmolyticus 4A and II R.—Neither of these organisms grew perceptibly on our medium although they were still alive at the end of 14 days. Glycerol was not consumed; acid was not produced. About 85 per cent of the histidine originally introduced was recovered in each case. The initial ammonia concentration was reduced by only 10 per cent. Histamine and other imidazoles were not formed.

The results obtained with a strain of *Streptococcus viridans* were quantitatively almost identical with those obtained above.

Pneumococci, Types I, II, III, and IV.—These organisms grew poorly on our medium. They removed 15 to 20 per cent of the histidine originally introduced in such a way that the nitrogen cannot be accounted for either as NH_3 or as NH_2. There is no evidence that a nuclear rupture occurred. The constancy of the ammonia value indicates strongly that these organisms do not use it as a source of nitrogen. Histamine and other imidazoles were not formed.

When leucine (0.10 gm.) was added to the medium the organisms multiplied rapidly for a few days. The addition of leucine had no effect upon the NH_3 and pH values and the histidine was removed to about the same extent as in the leucine-free medium. The leucine, however, was almost completely destroyed in every case. This would seem to indicate that leucine is a good source of nitrogen and carbon for the pneumococcus while histidine, ammonia, and glycerol are poor sources of these elements.

B. tuberculosis H.Sp., 1305, 3161, and O.H.—These four strains of tubercle bacilli gave results that were qualitatively identical though quantitatively somewhat different. In every case there is good evidence that imidazole acetic, propionic, lactic, or acrylic acids were present. The color obtained with the histidine fraction was too red for histidine. The values obtained for histidine by the amino nitrogen method were always decidedly lower than those obtained colorimetrically. The discrepancies between the color values and the amino nitrogen values are equal to 0.015 gm. (12.2 per cent) for H.Sp., 0.030 gm. (24.5 per cent) for No. 1305, 0.02 gm. (16.3 per cent) for No. 3161, and 0.023 gm. (18.7 per cent) for O.H., calculated as imidazole propionic acid. The organisms grew very well on the surface of our medium. An excess of acid was not produced during this time interval although the glycerol had been largely destroyed. The ammonia consumption was distinctly different in the four cases, varying from 40 per cent in the case of H.Sp. to only 10 per cent in the case of O.H. Histamine was not produced in any case.

A striking contrast to these four organisms was that of a fifth strain which is widely known as K_1. This organism has been grown on synthetic media for so long—about 40 years—that it has lost its virulence entirely. It grows excellently on our medium, the growth not being confined to the surface. Chemically it did not behave like a typical tubercle bacillus as can be seen by examining the last two horizontal columns of Table VIII. This organism attacked the histidine immediately and so effectively that 53 per cent of it had been destroyed at the end of 5 days of incubation. Some of the histidine nitrogen was converted into ammonia; some appeared as a non-volatile, carboxylated amine. After 45 days of incubation only 12 per cent of the histidine originally introduced remained. An excess of acid was not formed although the glycerol had been destroyed. Imidazole propionic acid and histamine were not produced.

In concluding this part of the work we wish to call special attention to the fact that these experiments have all been carried out in an artificial synthetic medium. Our conditions are, at

best, highly artificial and the composition of our synthetic medium is far simpler than that of living tissue or even than that of the usual composite culture media. We are well aware that results obtained in such a medium must be applied carefully, if at all, to the general problem of amine production in the living organism. We feel, however, that our mode of procedure is justified by the fact that we have first to develop methods for estimating imidazoles and phenols under simple conditions before we can hope to estimate these substances in complex mixtures. The final answer to the question, "which microorganisms are probable amine producers in the human organism" can only be given after a medium has been used that contains all of the essential ingredients of a tissue or tissue extract. How definitely the amine production is influenced by the composition of the medium is shown clearly in the Part IV of this report where other amino-acids or peptones were added to our synthetic medium.

PART IV.

The Production of Histamine by Bacillus coli cystitis when Other Amino-Acids Are Present in the Medium Together with Histidine.

This investigation was undertaken to ascertain which amino-acids when added to our medium, augment the production of histamine and the growth of the microorganisms. The colon bacillus used in this work (*Bacillus coli cystitis*) was the strain employed by us in our original investigation.[1] We have found that this organism will always convert approximately 50 per cent of the histidine originally introduced into histamine when precautions are taken to have the initial pH of the medium 7.3 and to maintain a uniform temperature of 37°.

In the first experiment of this series we compared the carboxylase activity on our standard medium with that on a medium containing leucine and histidine in one case, and peptone and histidine in the other. The results obtained are summarized in Table IX.

The results obtained in the medium containing histidine as the only amino-acid call for very little special comment because they closely resemble those obtained and reported 2 years ago. In

TABLE IX—*Effect of Leucine and Peptone on the*

Composition of the medium.	Total color value of the test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Histidine dichloride, 0.2 gm. Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Match perfect. Color develops promptly. 112%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. 40%	0.85 cc. N_2 at 20° and 750 mm. 0.097 gm. histidine dichloride. 48.5%
Histidine dichloride, 0.2 gm. Leucine, 0.1 " Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. Color develops promptly. 103%	0.50 cc. = 4.5 mm. 1.00 " = 9.0 " Match poor. Color too yellow. 9.0%	0.72 cc. N_2 at 21° and 753 mm. 0.0822 gm. histidine dichloride. 41.1%
Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Match perfect. Color develops promptly. 112%	0.20 cc. = 3.0 mm. 0.40 " = 6.0 " Match good. 15%	0.65 cc. N_2 at 22° and 754 mm. 0.074 gm. histidine dichloride. 37%

* Colors matched against the (CR-MO) standard.

roduc ion of Histamine by Bacillus coli (cystitis).

Color value of the histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction. Before incubation.	Reaction. After incubation.
		cc.	*pH*	*pH*
05 cc. = 13.8 mm. 10 " = 27.5 " olor developed like that of histamine. 092 gm. of histamine dichloride in entire test solution. % of histidine converted into histamine.	1.04 cc. N_2 at 20° and 754 mm. 0.0964 gm. histamine dichloride. 59.8%	27 Hence the ≈ of 9 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.4
olor too intense to run directly; hence 10 cc. were diluted to 100 cc. Of this solution 20 cc. = 7.6 mm. 40 " = 15.2 " olor develops like that of histamine. 1275 gm. of histamine dichloride in entire test solution. % of histidine converted into histamine.	2.55 cc. N_2 at 22° and 744 mm. 0.231 gm. histamine dichloride. 143%	25 Hence the ≈ of 11 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.5
olor too intense to run directly; hence 10 cc. were diluted to 100 cc. Of this solution 25 cc. = 9.3 mm. 50 " = 18.5 " lor develops like that of histamine. 25 gm. of histamine dichloride n entire test solution. 4% of histidine converted into histamine.	1.45 cc. N_2 at 23° and 755 mm. 0.133 gm. histamine dichloride. 82.4%		7.3	5.2

this case 57 per cent of the histidine originally introduced was converted into histamine. The agreement between the values obtained colorimetrically and by the amino nitrogen method is good.

Although the organisms multiplied rapidly in the above medium, they grew far better in a medium containing leucine or peptone together with the histidine. When leucine was present, 79 per cent of the histidine originally introduced was converted into histamine, as determined from the colorimetric reading an increase of 22 per cent over the leucine-free medium. The amino nitrogen value is obviously much too high to represent histamine alone. A portion of the leucine may have been converted into isoamylamine which would appear together with histamine in the amyl alcohol extract and thus raise the amino nitrogen value of the histamine fraction.

In the presence of peptone, 77 per cent of the histidine originally introduced was converted into histamine; hence leucine and peptone are about equivalent in their ability to promote histamine formation. In this case the check between the colorimetric and amino nitrogen values is sufficiently close to warrant the conclusion that other amines were not formed in appreciable quantities. In this case the acidity was also neutralized less perfectly than in the case of leucine where a large amount of amine production, other than histamine, was indicated.

In short then, we would conclude that the presence of either leucine or peptone stimulated the production of histamine. We can draw no conclusions from this experiment, as to the rôle played by the leucine or peptone in the amine production. We might conclude, with Jacoby,[9] that leucine was an easily available source from which carboxylase enzymes could be synthesized; but our experiment seems to indicate that the chief factor is the enormous increase in the speed of growth of the microorganisms which would, of course, increase the rate of acid production and hence render the early formation of histamine necessary to neutralize the acid.

We have grown *all* of the organisms discussed in the first three sections of this paper on a medium containing both leucine and histidine and have found that:

[9] Jacoby, M., *Biochem. Z.*, 1917, lxxxi, 332; lxxxiii, 74; lxxxiv, 358; 1918, lxxxvi, 329.

1. The addition of leucine always facilitates the growth of the organisms.

2. If the organisms produce no histamine in the absence of leucine, they will not produce histamine when leucine is present.

3. If the organisms produce histamine when leucine is absent, they will always produce 20 to 25 per cent more of this amine, in 2 weeks of incubation, when leucine is present. The leucine augments a power that already exists; it does not call forth a new enzymatic activity.

The second series was an enlargement upon the first for in this case all of the easily available amino-acids were employed; namely, glycine, alanine, cystine, leucine, arginine, glutamic acid, tyrosine, and tryptophane. In addition to the above amino-acids two flasks were prepared containing different makes of peptone; namely, Witte and Difco. The results obtained are summarized in Table X.

Table X shows that the histamine value on our standard medium was 20 per cent lower this time than it has been in any of our other experiments with this organism. The value on the leucine-histidine medium is also 20 per cent low. All of the flasks of *this* series were incubated at the same time in the same incubator. During the first few days the air in the incubator smelled strongly of hydrogen sulfide which was being evolved from the cystine-containing flask. We feel convinced that the retardation in speed of activity, and probably in rate of growth, was caused by the hydrogen sulfide. This conclusion is strengthened by the fact that only 2 per cent of histamine was formed in the cystine-histidine medium. The growth in this medium was also very meager. Since this hydrogen sulfide interference must have been approximately equivalent for all of the media, we believe that the values obtained are accurate in so far as they may be compared with one another. In discussing this series, a conversion of 31 per cent of the histidine originally introduced into histamine is considered to be the normal conversion on our standard medium. When more than 31 per cent of histamine was present, the amino-acid added can be considered to be an amine production *stimulator*, and *vice versa*. The amino nitrogen values on both the histidine and histamine fractions have, in every case, been calculated as histidine and as histamine, respectively, in spite of the fact

TABLE X—*Effect of Amino-Acids and Peptones*

Composition of the medium.	Histidine dichloride, 0.2 gm. KNO3, 0.1 " NH4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Glycine, 0.065 " KNO3, 0.1 " NH4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Alanine, 0.16 " KNO3, 0.1 " NH4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Leucine, 0.115 " KNO3, 0.1 " NH4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 Cystine, 0.2 KNO3, 0.1 NH4Cl, 0.2 Glycerol, 4.0 Usual inorganic salts. Distilled water to 200
Total color value calculated as % of histidine dichloride. (0.20 gm. = 100%), *per cent.*	112	108	100	112	96
Unchanged histidine colorimetric method. (0.20 gm. = 100%), *per cent.*	60	43	30	37	87
Amino nitrogen value of histidine fraction calculated as % of histidine dichloride. (0.20 gm. = 100%), *per cent.*	74	102.5	154	55	147
Histidine converted into histamine (colorimetric method) *per cent.*	31	37.2	51	49.6	2.06
Amino nitrogen value of histamine fraction calculated as % of histamine dichloride, *per cent.*	32	49	70.7	152	

Production of Histamine by Bacillus coli (cystitis.)

tidine ichlor- le, 0.2 gm. tamic id Cl, 0.16 " O_3, 0.1 " $_4$Cl, 0.2 " cerol, 4.0 cc. al in- ganic lts. tilled ater to 200 "	Histidine dichlor-ide, 0.2 gm. Arginine carbon-ate, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in-organic salts. Distilled water to 200 "	Histidine dichlor-ide, 0.2 gm. Tyrosine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in-organic salts. Distilled water to 200 "	Histidine dichlor-ide, 0.2 gm. Trypto-phane, 0.18 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in-organic salts. Distilled water to 200 "	Histidine dichlor-ide, 0.2 gm. Peptone (Witte), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in-organic salts. Distilled water to 200 "	Histidine di-chloride, 0.2 gm. Peptone (Difco), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in-organic salts. Distilled water to 200 "
105	110		99	103	97
62	52	11.5	60	40	50
115.5	109.8	10.6	79.5	60.6	67.1
21.7	41	31.4	26.3	42	42
42.5	108.4			56.6	62.8

TABLE

Composition of the medium.	Histidine dichloride, 0.2 gm. KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Glycine, 0.065 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Alanine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Leucine, 0.115 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 g Cystine, 0.2 KNO_3, 0.1 NH_4Cl, 0.2 Glycerol, 4.0 Usual inorganic salts. Distilled water to 200
0.10 N HCl neutralized by NH_3 from the entire test solution, *cc.*	20.5 Hence the ≈ of 16 cc. of 0.1 N NH_3 was used by microorganisms.	16.5 Hence the ≈ of 19.5 cc. of 0.1 N NH_3 was used by microorganisms.	17 Hence the ≈ of 19 cc. of 0.1 N NH_3 was used by microorganisms.	23.7 Hence the ≈ of 12.3 cc. of 0.1 N NH_3 was used by microorganisms.	9.2 Hence the of 26.8 of 0.1 NH_3 w used by microorgan-isms.
pH after 14 days of incubation. The initial pH was 7.3 in all cases, *pH.*	5.5	5.5	5.6	5.9	5.3
Tyrosine converted into volatile phenols (colorimetric method), *per cent.*					
Tyrosine converted into aromatic hydroxy-acids. Calculated as oxyphenyllactic acid (colorimetric method) *per cent.*					
Unchanged tyrosine (colorimetric method), *per cent.*					
Tyrosine converted into tyramine (colorimetric method) *per cent.*					

ɜtidine lichlor- de, 0.2 gm. tamic id Cl, 0.16 " O₃, 0.1 " Cl, 0.2 " cerol, 4.0 cc. al in- ganic lts. tilled ıter to 200 "	Histidine dichlor- ide, 0.2 gm. Arginine carbon- ate, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in- organic salts. Distilled water to 200 "	Histidine dichlor- ide, 0.2 gm. Tyrosine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in- organic salts. Distilled water to 200 "	Histidine dichlor- ide, 0.2 gm. Trypto- phane, 0.18 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in- organic salts. Distilled water to 200 "	Histidine dichlor- ide, 0.2 gm. Peptone (Witte), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in- organic salts. Distilled water to 200 "	Histidine di- chloride, 0.2 gm. Peptone (Difco), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual in- organic salts. Distilled water to 200 "
17.5 nce the ≈ of 18.5 cc. of 0.1 N NH_3 was used by microorganisms.	19.5 Hence the ≈ of 16.5 cc. of 0.1 N NH_3 was used by microorganisms.	23.5 Hence the ≈ of 12.5 cc. of 0.1 N NH_3 was used by microorganisms.	20 Hence the ≈ of 16 cc. of 0.1 N NH_3 was used by microorganisms.	24.5 Hence the ≈ of 11.5 cc. 0.1 N NH_3 was used by microorganisms.	22.2 Hence the ≈ of 13.8 cc. of 0.1 N NH_3 was used by microorganisms.
5.9	5.6	5.7	5.4	5.5	5.4
		None.			
		3.4			
		72.6			
		None.			

that the values must obviously be high because of the presence of the other amino-acid or peptone. These amino nitrogen figures are of value because they give some information about the fate of the added amino-acid.

Glycine and Histidine.—The organisms grew distinctly better in a medium containing glycine and histidine than they did in a medium containing only histidine. The histamine production was increased by 6.2 per cent. Most of the glycine nitrogen remained in the histidine fraction in the primary amino condition which indicates that only a small amount of this amino-acid was utilized by the microorganisms.

Arginine and Histidine.—The microorganisms grew very much better in this medium than they did in the one containing glycine and histidine. The histamine production was increased by 10 per cent. The amino nitrogen figures indicate that over half of the arginine was converted into some product that passed into amyl alcohol from a strongly alkaline solution. The arginine may, therefore, also have been decarboxylated.

Peptone and Histidine (Witte and Difco).—The presence of either of the above peptones is a great stimulus to the rate of growth of these bacilli, the growth being about equivalent to that obtained in the arginine-histidine medium. The histamine production was increased by 11 per cent in each case.

Alanine and Histidine.—The organisms grew even better in a medium containing alanine than they did in one containing peptone. In this case the histamine production was increased by 20 per cent, the highest value obtained with any of the amino-acids. The alanine used in this work was a mixture of equal parts of the *d*- and *l*-varieties. Two equivalents of this alanine were used. It is interesting to note that one and a quarter equivalents remained, probably as alanine, in the histidine fraction and that a maximum of one-fifth of an equivalent passed into amyl alcohol, possibly as an amine. In short, then, over half of the introduced available nitrogen was consumed by the microorganisms. (We have assumed that *l*-alanine did not serve as a food.)

Leucine and Histidine.—The growth obtained in this case was slightly better than that obtained on the histidine-alanine medium. The histamine production was increased by 18.6 per cent which is almost identical with that obtained on the alanine-histidine

medium. Most of the leucine nitrogen appeared in the histamine fraction which indicates that the leucine was also decarboxylated.

Tryptophane and Histidine.—The growth in this medium was about as dense as that obtained in the peptone-histidine medium; but the histamine production was *decreased*, not increased. The 26.3 per cent of histamine obtained in this case is about 5 per cent less than that obtained in a medium containing histidine as the only amino-acid. We anticipated some trouble with our colorimetric process in this case; but we were relieved to find that tryptophane, or its decomposition products, did not interfere in any way with the color production.

Glutamic Acid and Histidine.—The growth on this medium was slightly less than that on the tryptophane-histidine medium. The presence of glutamic acid *decreases* the histamine production by about 9 per cent. The amino nitrogen figures indicate that most of the glutamic acid nitrogen was still attached to a carboxylated molecule.

Cystine and Histidine.—The growth on this medium was very meager. The presence of cystine decreases the histamine production by 29 per cent so that the formation of this amine is almost nil.

Tyrosine and Histidine.—The fact that tyrosine and its derivatives give a color with alkaline *p*-phenyldiazonium sulfonate, made it necessary for us to modify our usual procedure in this case. The method employed was briefly as follows:

1. The filtered medium, after the usual treatment with 1 cc. of 95 per cent sulfuric acid, was subjected to a distillation, under ordinary pressure, the distillate being collected and examined colorimetrically[10] for volatile phenols. Phenols were absent.

2. The contents of the distilling flask were transferred to a glass dish and evaporated on the water bath. The residue was transferred, with water, to a 25 cc. precision cylinder and diluted to 25 cc. This is the *test liquid.*

3. Of this acid *test liquid* 10 cc. were transferred to a 35 cc. extraction bottle and extracted ten times with ether, using 20 cc. for each extraction. The combined ether extracts were treated

[10] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 235.

with 25 cc. of water, the ether being then removed by distillation at first under ordinary pressures and then *in vacuo*. The solution so obtained was transferred, with water, to a 100 cc. precision cylinder and diluted to 100 cc. It was tested, colorimetrically,[10] for aromatic hydroxy-acids. In this way 3.4 per cent of the tyrosine originally introduced was found to have been converted into oxyphenyllactic acid.

4. The aqueous liquid obtained above, that had been freed from aromatic hydroxy-acids by extraction with ether, was transferred to a 250 cc. Pyrex flask with 90 cc. of water. Silver nitrate, 10 cc. of a 20 per cent solution, was added and the resulting mixture treated with 12 gm. of barium hydroxide in 50 cc. of warm water. The dark brown mixture was filtered, the precipitate being washed with a cold saturated solution of baryta.[11]

This divides the material into two fractions; *the silver precipitate*, which will contain all of the histamine and which should contain most of the histidine, and *the silver filtrate* which should contain all of the tyrosine and tyramine.

5. *The silver precipitate* was suspended in water and treated with an excess of 37 per cent HCl and Na_2SO_4 as has been previously described.[11] The resulting mixture was filtered after 2 hours of digestion on the water bath and the filtrate evaporated on the water bath. The residue was then transferred to an extraction bottle with 10 cc. of water and treated just like any of the histidine test liquids. It was found to contain 11.5 per cent of histidine and 31.4 per cent of histamine. The recovery of imidazoles was only 43 per cent. We have not checked up on the silver precipitation method for histidine sufficiently to be certain that some histidine may not have remained unprecipitated. We are certain that the histamine figure is accurate because the method employed in this experiment was identical with that used in the quantitative precipitation of histamine in some of our earlier work.[11]

6. *The silver filtrate.* The barium and silver ions were exactly removed with H_2SO_4 and HCl, respectively. The filtrate from $BaSO_4$ and AgCl was neutralized with sodium hydroxide and evaporated on the water bath. The residue was transferred to

[11] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.

an extraction bottle with 10 cc. of water. The subsequent separation of tyramine from tyrosine and the colorimetric estimation of each substance in its respective fraction was then carried out as described in the following paper.[12] Tyramine was found to be absent. Of the tyrosine originally introduced, 72.6 per cent was recovered.

The histamine production on this medium proceeded at a rate identical with that on a tyrosine-free medium. *Tyrosine neither augmented nor retarded the histamine formation. Tyrosine was not decarboxylated.*

It appears then, that the decarboxylation of histidine is influenced by the presence of other amino-acids in all of the three possible ways. *Tyrosine is without effect. Leucine, alanine, arginine, and glycine increase the rate of decarboxylation. Of these, leucine and alanine are by far the most efficient. Cystine, glutamic acid, and tryptophane decrease the rate of decarboxylation. Of these, cystine is by far the most efficient.* The rate of decarboxylation is not entirely coincident with the rate of growth of the microorganisms because, with the exception of cystine, all of the amino-acids augmented the growth of the organisms; but tryptophane and glutamic acid *decreased* the histamine production.

SUMMARY.

1. The behavior of a large number of microorganisms has been studied on a liquid medium consisting of histidine dichloride (0.2 gm.), ammonium chloride (0.2 gm.), potassium nitrate (0.1 gm.), potassium dihydrogen phosphate (0.4 gm.), sodium chloride (0.8 gm.), sodium sulfate (0.02 gm.), sodium bicarbonate (0.4 gm.), calcium chloride (0.01 gm.), and glycerol (4.0 cc.), in a total aqueous volume of 200 cc.

2. The organisms studied were *Bacillus coli communior* (7 strains), *Bacillus coli communis* (5 strains), *Bacillus lactis aerogenes* (5 strains), *Bacillus acidi lactici* (12 strains), *Bacillus enteritidis, Bacillus typhosus, Bacillus paratyphosus* A (3 strains), *Bacillus dysenteriæ* Flexner, Morgan, and Shiga, *Bacillus fæcalis alcaligenes* I and III, *Bacillus mucosus capsulatus* (3 strains), *Bacillus bifidus, Bacillus influenzæ, Bacillus proteus vulgaris* (2 strains), *Bacillus cloacæ, Streptococcus hæmolyticus* and *viridans*, pneumococci (Types I, II, III, and IV), and *Bacillus tuberculosis* (5 strains).

[12] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 193.

3. *Of the twenty-nine strains of coli*—in the narrower sense—that we studied, *six* were able to convert histidine into histamine on our synthetic medium. None of the other organisms were histamine producers.

4. Five members of the colon group—in the narrower sense—gave quantitative evidence that an alkali-stable, carboxylated triamino compound was formed from the histidine. This compound may have the formula

$$\begin{array}{l} \mathrm{HC - NH_2} \\ \quad \| \\ \mathrm{HC - NH_2} \\ \quad | \\ \quad \mathrm{CH_2} \\ \quad | \\ \mathrm{HCNH_2} \\ \quad | \\ \quad \mathrm{COOH} \end{array}$$

None of the other organisms gave quantitative evidence for the formation of such a compound; but most of them gave results that would lead one to suppose that such a compound was formed to some extent as an intermediate in the decomposition of histidine.

5. Imidazole acetic, propionic, lactic, or acrylic acid was formed by *Bacillus paratyphosus* A (1 strain), *Bacillus dysenteriæ* Flexner, Morgan, and Shiga, *Bacillus fæcalis alcaligenes* I, *Bacillus mucosus capsulatus* (2 strains), and *Bacillus tuberculosis* (4 strains). We cannot say for certain which of the above acids was produced in any case because our method does not differentiate between them.

6. The addition of leucine to the standard medium facilitates the growth of all of the organisms studied.

7. If the organisms produce no histamine when leucine is absent, they do not produce histamine when leucine is present.

8. If the organisms produce histamine when leucine is absent, they produce 20 to 25 per cent more of this amine, in 2 weeks of incubation, when leucine is present. The leucine augments a power that already exists; it does not create a new enzymatic activity.

9. The addition of alanine, leucine, arginine, glycine, or peptone—either Witte or Difco—to the standard medium, augments the growth of the colon bacillus and increases the yield of histamine.

When glutamic acid or tryptophane are added to the standard medium, the growth of the organisms is augmented; but the output of histamine is decreased. Cystine is unfavorable to the growth of the colon bacillus. The presence of this amino-acid reduces the yield of histamine to almost nil. The remarkable retarding influence of cystine on the growth of the microorganisms and on the histamine production by the colon bacillus seems to be due to the hydrogen sulfide which is evolved in quantity during the first few days of incubation. The addition of tyrosine to our standard medium seems to have no influence upon the rate of histamine production.

We wish to acknowledge the technical assistance of Miss Edith H. Bell throughout this work.

STUDIES ON PROTEINOGENOUS AMINES.

XIII. ON THE ELECTRONIC INTERPRETATION OF CERTAIN BIOCHEMICAL PHENOMENA.

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CONTENTS.

In our work on biological problems we have frequently encountered reactions that could not have been readily explained on the basis of the accepted structural formulas of the compounds involved, because these formulas are incomplete indices of the degree of oxidation of the carbon atoms. We were led, therefore, to a consideration of the interatomic forces and we have found that the electronic formulas so derived have been of great service in clarifying certain puzzling phenomena.

Parts I and II of this paper contain an electronic explanation of the carboxylase activity of yeast and of the fact that the fatty acids oxidize predominantly in the beta position in the animal body. In Part III we will attempt to show how the electronic formulas for some of the biologically important compounds can be derived.

PART I.

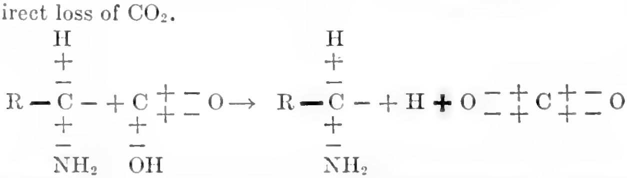
On the Carboxylase Activity of Microorganisms with Particular Reference to Yeasts.

One of the main sources of the formation of amines by the living cell is the decarboxylation of amino-acids. While chiefly accomplished through the activity of microorganisms, there is evidence that this carboxylase activity is also part of the life process of some higher plants and of certain animals. The occurrence of *p*-oxyphenylethylamine in several species of mistletoe[1] (*Phoradendron flavescens*, *Phoradendron villosum*, and *Phoradendron californicum*) and the presence of the same amine in the salivary gland and secretion of some Cephalopoda[2] may be recalled in this connection as evidence for this statement. Up to the present time, however, enzymes capable of decarboxylating amino-acids to amines *in vitro* have not been isolated from either unicellular or multicellular organisms. While the isolation of such an enzyme of the carboxylase type would be of interest, it is doubtful if this accomplishment would clarify the mechanism of decarboxylation any more than the discovery of zymase has enhanced the problem of alcoholic fermentation and of the breakdown of sugars. To obtain a better understanding of the enzymatic activities of the living cell it would seem far more promising to undertake a systematic study of the intermediates formed in the catabolism of higher molecular complexes, especially if we could understand the interatomic forces which govern this breakdown.

The decarboxylation of amino-acids, with the formation of carbon dioxide and the corresponding amine might, theoretically, be accomplished in any of the following ways.

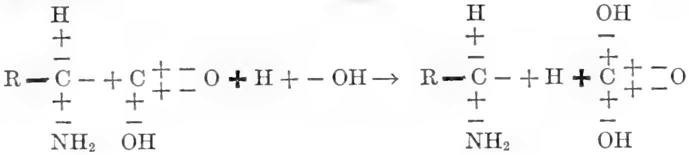
1. With a quadruply positive carboxyl group.

A. Direct loss of CO_2.

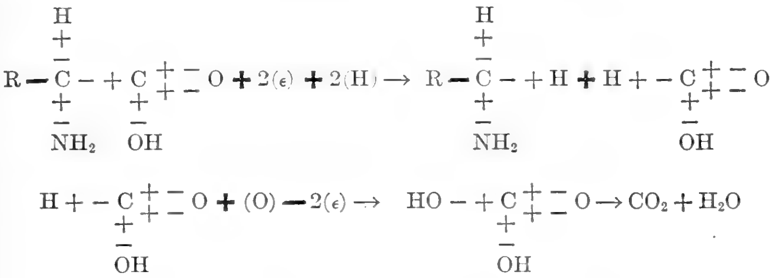


[1] Crawford, A. C., and Watanabe, W. K., *J. Biol. Chem.*, 1916, xxiv, 169.
[2] Henze, M., *Z. physiol. Chem.*, 1913, lxxxvii, 51.

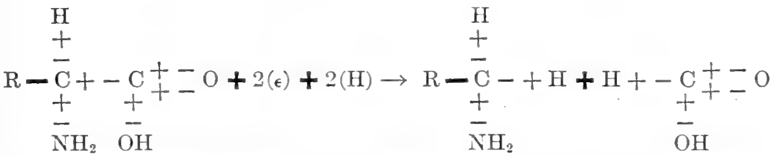
B. Hydrolysis.



C. Reduction of the quadruply positive carboxyl group.

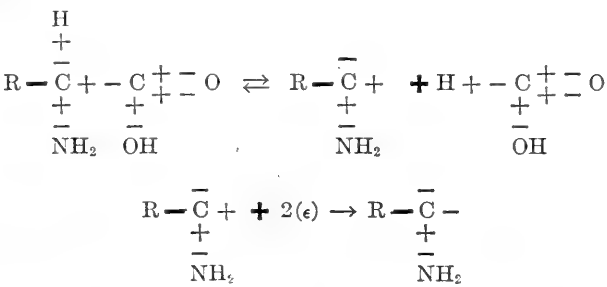


2. With a triply positive carboxyl group.



The formic acid would then give CO_2 and H_2O, by oxidation, as in C above.

The above equation tells nothing about the mechanism of the reaction. Following Nef, we might consider the above reaction to proceed as follows.[3]



[3] Koessler, K. K., *Proc. Inst. Med. Chicago*, 1920, iii, 46.

$$R - \underset{\underset{\underset{\displaystyle NH_2}{-}}{+}}{\overset{-}{C}} - \; + \; 2(H+) \rightarrow R - \underset{\underset{\underset{\displaystyle NH_2}{-}}{+}}{\overset{\overset{\overset{\displaystyle H}{+}}{-}}{C}} - + H$$

For reasons that will be discussed later, the schematic possibility represented by equation (1, C) is hardly worthy of consideration because it involves the reduction of a quadruply positive carbon atom. As far as we now know, only the chlorophyll-containing plants have this power.

A consideration of Types I and II shows that the kind of reaction resorted to must depend upon the direction of the electrical field between the carboxyl group and its neighboring carbon atom. If we were certain that the carboxyl group in all amino-acids was negative with respect to its neighboring carbon atom as in formula (2), we could state with a fair degree of assurance that the decarboxylation of amino-acids must be associated with an oxidation reduction process. Unfortunately, we are at present unable to make a definite statement as to the electrical conditions that prevail in any amino-acid (see under allyl chloride, pages 231 to 233). It seems indeed, as if the charge on the carboxyl group is not the same for all amino-acids.

Although we are, therefore, unable at present to give an electronic interpretation of the carboxylase activity as applied to amino-acids, the electronic point of view offers a fascinating explanation for a related type of carboxylase activity.

Neuberg[4] and his coworkers have shown that yeasts and yeast extracts have the faculty of decarboxylating pyruvic and many other organic acids with the formation of carbon dioxide and aldehydes; *e.g.*, $CH_3-CO-COOH \rightarrow CO_2 + CH_3-CHO$.

Karczag and Breuer[5] have shown that many bacteria, although they ferment pyruvic acid with gas formation, produce no aldehyde. The gas produced, instead of being pure CO_2 as in the case of the yeasts, consists of hydrogen to the extent of 90 per

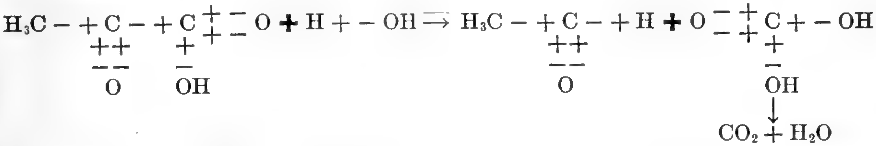
[4] Neuberg, C., and Hildesheimer, A., *Biochem. Z.*, 1911, xxxi, 170. Neuberg, C., and Tir, L., *Biochem. Z.*, 1911, xxxii, 323. Neuberg, C., and Karczag, L., *Biochem. Z.*, 1911, xxxvi, 68, 76; xxxvii, 170. Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xlvii, 413, 405.

[5] Karczag, L., and Breuer, E., *Biochem. Z.*, 1915, lxx, 320.

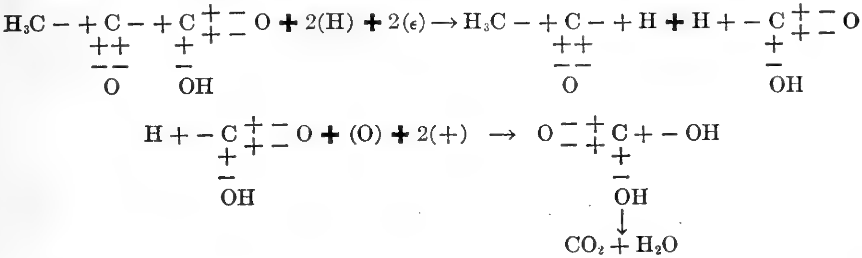
cent. Obviously, then, the action of yeasts on pyruvic acid is radically different from that of the investigated bacteria. The authors believe that some light is shed upon these facts and others to be presented later, by a consideration of the electronic formulas of these substances.

Acetaldehyde and carbon dioxide could be obtained from pyruvic acid by either of the three following processes.[6]

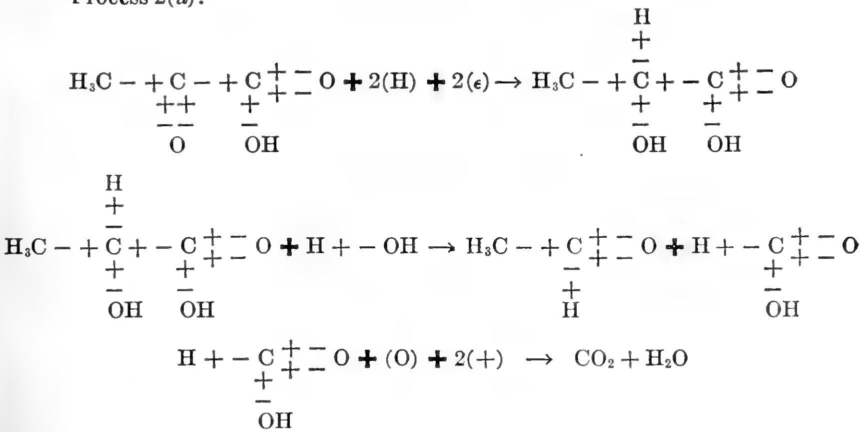
1. Hydrolysis.



2. Reduction followed by oxidation.



Process 2(a).



[6] The proof of the electronic formulas for the compounds used in the equations throughout this paper is given in Part III.

The decomposition of pyruvic acid by yeast *cannot* occur according to process (2) or (2, a) for the following reasons.

Process (2) demands the intermediate formation of formic acid *by the reduction of a quadruply positive carbon atom.* Such a reduction would be equivalent to the direct conversion of CO_2 to formic acid. Up to the present time only the chlorophyll-containing plants have been proved to be capable of reducing CO_2, and they can do so only in the presence of sunlight. This process is, therefore, highly improbable from the start. If we pass lightly over this first serious objection, we come to another that is just as serious. The formulation demands that the formic acid formed at first must then be oxidized to CO_2. That bacteria have this power of oxidation is clearly proved by the work of Omelianski[7] and Pakes and Jollyman.[8] In every case, however, hydrogen is evolved along with the CO_2. Hydrogen is never evolved in the early stages of yeast fermentations. Although the evolution of CO_2 from pyruvic acid by yeast is extremely rapid so that the fermentation is practically over in 24 hours irrespective of the variety of yeast employed, Neuberg and Tir[4] have shown that certain of these yeasts will evolve no gas at all from sodium formate solutions and that none of them evolves more than traces of CO_2 in the course of 24 hours.

An objection might be raised to these conclusions from the work of Neuberg and Tir because these authors used a pure solution of sodium formate in which no real fermentation could occur. It is conceivable that the oxidation of formic acid might take place only in the presence of sugar. Franzen and Steppuhn[9] have shown conclusively that when sodium formate is mixed with a nutrient medium rich in sugar, most of the pure cultures of yeast employed, although they fermented the sugar vigorously, either did not ferment the added formic acid at all or did so only to a very small extent. In no case was the destruction of the formic acid *rapid* enough to conclude that this substance was an intermediate product in the fermentation of sugar. Formulation (2) is, therefore, highly improbable.

[7] Omelianski, W., *Centr. Bakt., 2te Abt.*, 1904, xi, 177, 256, 317.

[8] Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, 1901, lxxix, 322, 386, 459.

[9] Franzen, H., and Steppuhn, O., *Z. physiol. Chem.*, 1912, lxxvii, 129.

Process (2, a) demands the intermediate formation of lactic acid. That the reduction of pyruvic acid to lactic acid might be resorted to by *bacteria* in case the pyruvic acid was being used by them as a source of carbon is very possible because as can be seen from the formula, this is an indirect but simple method for converting an *unavailable quadruply positive carboxyl group into one that is triply positive.* That yeasts do *not* resort to this process, in the case of pyruvic acid, is proved by the fact that *lactic acid is not fermented* at all by some yeasts and only to a very slight extent by most (see article by Neuberg and Tir[4] and Buchner and Meisenheimer[10]).

Even if lactic acid were fermented by yeast, the formic acid difficulty would still be insurmountable (see above under process (2)). Apparently then, formula (2, a) cannot represent what happens when pyruvic acid is fermented by yeast.

Process (1) is the only one left and its very simplicity is exactly what one would expect from a reaction that proceeds so smoothly and rapidly. The above considerations have convinced the authors that *the fermentation of pyruvic acid by yeast is accomplished by a purely hydrolytic process.*

From the ordinary structural formula for lactic acid it is difficult to see why a similar hydrolysis does not occur in this case with the formation of CO_2 and alcohol. The hydrolysis of lactic acid with *dilute sulfuric* acid, which takes place quite as readily as the similar hydrolysis of pyruvic acid, gives acetaldehyde and formic acid. The electronic formulas for these two compounds show clearly why CO_2 could not be obtained by the hydrolysis of lactic acid and they *show moreover why yeast cannot hydrolyze lactic acid at all.* Lactic acid contains a *triply* positive carboxyl group while pyruvic acid contains one that is *quadruply* positive. Apparently then, *yeast can hydrolyze only those acids containing quadruply positive carboxyl groups.*

With this idea in mind it is easy to see why oxalacetic acid should give acetaldehyde and CO_2, acetone dicarbonic acid should give acetone and CO_2, and in general why all of the α-ketonic acids should give an aldehyde and CO_2 as primary products because *they all contain quadruply positive carboxyl groups.* A striking confirmation of this statement is found in the fact that *glyoxylic*

[10] Buchner, E., and Meisenheimer, J., *Ber. chem. Ges.*, 1910, xliii, 1773.

acid, the mother substance of the α-ketonic acids, *is practically not fermented by yeast.*[4] *This acid has been shown to contain a negative carboxyl group and it could not give CO_2 on hydrolysis.*

No attempt has been made to explain the mechanism of hydrolysis. Just why pyruvic acid should hydrolyze readily whereas acetic acid must be fused with an alkali to hydrolyze it is not explained by the electronic formulas. The failure of yeast to ferment an acid does not prove that the acid contains a *negative* carboxyl group. On the other hand, if yeast does split CO_2 from an acid *readily* it is fairly safe to conclude that the acid contains a quadruply positive carboxyl group. Acetic acid is fermented very slowly by yeast. Neuberg and Tir make the statement that the gas evolved is only partially absorbed by sodium hydroxide. It would be of interest to ascertain if the unabsorbed gas was methane because if it was methane, the yeast would have proved itself capable of hydrolyzing even this very resistant acid.

PART II.

On the Beta Oxidation of Fatty Acids in the Animal Body.

The work of Knoop, Dakin, and others[11] leaves little doubt that fatty acids undergo oxidation predominantly in the beta position in the animal body. Just why the living cells and hydrogen peroxide should oxidize the beta carbon atom by preference while the halogens react only with the alpha hydrogen atoms of the fatty acids is a question that has been much discussed in the past. It occurred to the authors that this tendency toward beta oxidation in the body (and by hydrogen peroxide) might be closely associated with the electrical structure of the molecule.

The carbon atom can occur in five stages of oxidation; namely,

$$1.\ -\underset{-}{\overset{-}{C}}- \qquad 2.\ -\underset{-}{\overset{-}{C}}+ \qquad 3.\ -\underset{+}{\overset{-}{C}}+ \qquad 4.\ -\underset{+}{\overset{+}{C}}+ \qquad 5.\ +\underset{+}{\overset{+}{C}}+$$

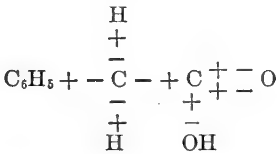
Carbon dioxide is the simplest substance that contains carbon in the completely oxidized condition represented by form (5). Neither microorganisms nor the cells of the animal organism seem to have the faculty of reducing CO_2. Only the chlorophyll-

[11] The references to the original literature are given in Dakin's monograph (Dakin, H. D., Oxidation and reductions in the animal body, London, 1912).

containing plants have been proved to be capable of reducing CO_2 to formaldehyde and they can do so only in the presence of sunlight.[12] Forms (2), (3), and (4) seem to be oxidizable or reducible by living matter depending upon the conditions of the experiment. At present it seems advisable to make no definite statement as to which of these forms is most readily oxidizable.

The work of numerous investigators seems to indicate that form (1) is not *readily* oxidized by living matter. Thus Karczag and Breuer[5] showed that oxalacetic, acetone dicarbonic, and acetic acids were not changed by a large number of microorganisms. Of these acids, acetone dicarbonic and acetic acids contain only quadruply negative and quadruply positive carbon. Oxalacetic acid has one quadruply negative, two quadruply positive, and one triply positive carbon atom. From this it would appear that quadruply negative carbon was not readily oxidized by bacteria.

Although acetic, acetoacetic, and malonic acids, all of which contain only quadruply negative and positive carbon, are completely oxidized in the normal animal body, acetone is attacked with difficulty[13] and phenylacetic acid is not oxidized at all.[14] Acetone has been proved to contain only quadruply positive and quadruply negative carbon and phenylacetic acid must have the formula



It seems therefore, that even the *normal* body cells frequently find it *difficult* to oxidize quadruply negative carbon. From these facts two conclusions can be drawn.

1. If a compound containing quadruply negative carbon together with other more easily oxidizable varieties were fed to a *normal* animal one would expect a predominant and initial oxidation of the more easily oxidizable carbon atoms. In the case

[12] For a critical review of this subject see Bayliss, W. M., Principles of general physiology, London, New York, Bombay, Calcutta, and Madras, 1915, 564.

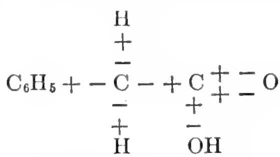
[13] Geelmuyden, H. C., *Z. physiol. Chem.*, 1897, xxiii, 431.

[14] Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 150.

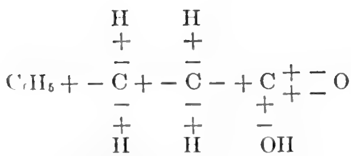
of *most* compounds, these primary oxidation products would then undergo further oxidation, because of the vigorous oxidizing conditions that prevail in the normal organism, so that an excretion of more than traces of these partially oxidized products is hardly to be expected.

2. In certain diseases, notably diabetes, the oxidizing power of the body cells is greatly diminished. Under these conditions the difficultly oxidizable quadruply negative carbon atoms might escape oxidation, at least partially, while the more easily oxidizable carbon atoms in the same molecule might be oxidized. These partially oxidized products, with the quadruply negative carbon still intact, should then be excreted. It is a well known fact that acetone and acetoacetic acid are excreted in large quantities by diabetics. One would expect also that acetic acid, acetone dicarbonic acid, citric acid, and a large number of other substances containing quadruply negative carbon should be at least partially excreted unchanged by diabetics. Citric acid contains one triply positive carbon atom. It might, therefore, be eliminated as acetone dicarbonic acid or if the carboxylase activity was too great it should surely appear as acetone. (The carboxylase of yeast readily decomposes acetone dicarbonic acid into carbon dioxide and acetone by a purely hydrolytic process as has been shown in Part I. A similar decomposition might occur in diabetics.)

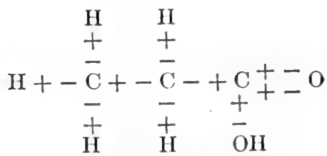
With the idea in mind that quadruply negative carbon is not readily oxidized by the body cells, we can now consider the probable primary oxidation products of a few acids whose electronic formulas shall be repeated here for the sake of simplicity.



(Phenylacetic acid.)



(Phenylpropionic acid.)



(Propionic acid.)

$$\begin{array}{lcccc}
 & H & H & H & \\
 & + & + & + & \\
 & - & - & - & \\
C_6H_5 + & -C- & +C+ & -C- & +C\,{}^{+}_{+}\,{}^{-}_{-}\,O \\
 & - & - & - & + \\
 & + & + & + & - \\
 & H & H & H & OH
\end{array}
\qquad
\begin{array}{lcccc}
 & H & H & H & \\
 & + & + & + & \\
 & - & - & - & \\
H + & -C- & +C+ & -C- & +C\,{}^{+}_{+}\,{}^{-}_{-}\,O \\
 & - & - & - & + \\
 & + & + & + & - \\
 & H & H & H & OH
\end{array}$$

(Phenylbutyric acid.) (Butyric acid.)

Phenylacetic acid contains the perfectly stable quadruply positive carboxyl group, the very resistant benzene ring, and the presumably difficultly oxidizable quadruply negative alpha carbon atom. This compound should, therefore, not be readily oxidized even by the normal body cells. As a matter of fact phenylacetic acid is *not* oxidized by the normal body cells.

Phenylpropionic acid is *partially oxidized only in the beta position.* This triply negative carbon atom should be the most vulnerable position in the molecule. An oxidation at this point would lead finally to benzoylacetic acid which by one type of hydrolysis would give benzoic acid and acetic acid. Knoop found that phenylpropionic acid was converted into benzoic acid in the normal animal while Dakin was also able to isolate a small amount of benzoylacetic acid under similar conditions.

Phenylbutyric acid *is partially oxidized only in the beta position.* This doubly positive carbon atom should be the most easily attacked position in the molecule. An oxidation at this point would lead to the formation of phenylacetoacetic acid which by one type of hydrolysis would give phenylacetic acid and acetic acid. Knoop found that phenylacetic acid was formed from phenylbutyric acid in the normal animal.

In propionic acid *it is again the beta position that is partially oxidized* and therefore, should be most readily still further oxidized. The final product of the oxidation would be malonic acid. Malonic acid is, however, very readily oxidized by the normal body cells; hence the isolation of a definite end-product is hardly to be expected in the normal animal. As a matter of fact propionic acid is completely oxidized in the normal animal without leaving a clue as to the mode of its decomposition. In diabetics, malonic acid, a polymer of the half aldehyde of malonic acid, or a condensation product of this half aldehyde might possibly be found after feeding propionic acid; but these products seem not to have been sought for.

Butyric acid *is partially oxidized only in the beta position.* Here too, this should be the most vulnerable position. A further oxidation at this point would lead finally to acetoacetic acid which by hydrolysis could lead either to acetone and carbon dioxide or to acetic acid. The increased excretion of acetoacetic acid and acetone after feeding butyric acid to diabetics is a well established fact.

The cited examples would lead one to conclude that the established fact that fatty acids oxidize predominantly in the beta position in the animal body, besides having a chemical verification is the fact that the same type of oxidation occurs with hydrogen peroxide, is exactly what one would expect from the electronic formulas of the fatty acids and their derivatives.

PART III.

Proof of the Electronic Formulas of a Few Biologically Important Organic Compounds.

The General Principles Used in the Determination of Electronic Formulas.

Principle I. The Polarity of a Double or Triple Bond.[15]—Consider an olefine derivative having the following structural formula:

$$\mathrm{A}-\overset{\mathrm{H}}{\mathrm{C}}=\overset{\mathrm{H}}{\mathrm{C}}-\mathrm{B}$$

[15] Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1910, xxxii, 1637. Stieglitz has presented this matter in lecture form for the past 6 years, and has applied it to the derivation of the complete formulas for the vinyl derivatives, including acetaldehyde, and for the partial formulas of many organic compounds such as propylene, acrylic acid, crotonic acid, and cinnamic acid. The formula so derived for the vinyl derivatives is $\mathrm{H}+-\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}\begin{smallmatrix}-+\\-+\end{smallmatrix}\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}+-\mathrm{X}$; for acetaldehyde is $\mathrm{H_3C}-+\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}\begin{smallmatrix}+-\\+-\end{smallmatrix}\mathrm{O}$; for propylene is $\mathrm{H_3C}-\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}\begin{smallmatrix}+-\\+-\end{smallmatrix}\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}-+\mathrm{H}$; for crotonic acid is $\mathrm{CH_3}-\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}\begin{smallmatrix}+-\\+-\end{smallmatrix}\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}-\mathrm{COOH}$; and for cinnamic acid is $\mathrm{C_6H_5}-\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}\begin{smallmatrix}+-\\+-\end{smallmatrix}\underset{\substack{-\\+\\\mathrm{H}}}{\mathrm{C}}-\mathrm{COOH}$

The following three electronic formulas are possible.

$$A - \overset{H}{C} \genfrac{}{}{0pt}{}{+\,-}{+\,-} \overset{H}{C} - B \qquad A - \overset{H}{C} \genfrac{}{}{0pt}{}{+\,-}{-\,+} \overset{H}{C} - B \qquad A - \overset{H}{C} \genfrac{}{}{0pt}{}{-\,+}{-\,+} \overset{H}{C} - B$$

Formula 1. Formula 2. Formula 3.

Which of these is the correct formula can be ascertained by determining how the olefine absorbs compounds of accepted polarity such as $H+-OH$, $H+-Cl$, $H+-Br$, $H+-I$, $H+-NHR$, and $H+-OR$.

A compound having formula (1) will absorb HBr, for example, to give

$$\begin{array}{ccccc} & H & & H & \\ A - & C & + - & C & - B \\ & + & & - & \\ & - & & + & \\ & Br & & H & \end{array} \text{ exclusively.}$$

A compound having formula (2) will absorb HBr to give a mixture of

$$\begin{array}{ccccc} & H & & H & \\ A - & C & + - & C & - B \\ & - & & + & \\ & + & & - & \\ & H & & Br & \end{array} \text{ and } \begin{array}{ccccc} & H & & H & \\ A - & C & - + & C & - B \\ & + & & - & \\ & - & & + & \\ & Br & & H & \end{array}$$

A compound having formula (3) will absorb HBr to give

$$\begin{array}{ccccc} & H & & H & \\ A - & C & - + & C & - B \\ & - & & + & \\ & + & & - & \\ & H & & Br & \end{array} \text{ exclusively.}$$

Principle II. The Determination of the Charge on a Carboxyl Group.—Three methods have been employed.

1. In a few cases ketenes are known that have the general formula $R-C\genfrac{}{}{0pt}{}{-}{-}C\genfrac{}{}{0pt}{}{-}{-}O$. They always have the electronic formula $R-C\genfrac{}{}{0pt}{}{-\,+}{-\,+}C\genfrac{}{}{0pt}{}{+\,-}{+\,-}O$ because they yield acids, anilides, amines, and esters when treated with water, aniline, ammonia, and alcohol, respectively. This is an application of Principle I. In such cases the carboxyl group is quadruply positive.[16]

2. Many carboxylated compounds lose CO_2 when heated. Since CO_2 must have the formula $O\genfrac{}{}{0pt}{}{-\,+}{-\,+}C\genfrac{}{}{0pt}{}{+\,-}{+\,-}O$, such compounds contain a quadruply positive carboxyl group.[17]

[16] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1730.
[17] Fry, H. S., *J. Am. Chem. Soc.*, 1912, xxxiv, 664; 1914, xxxvi, 248.

3. Some carboxylated compounds split off formic acid when they are heated to 100–150° with dilute sulfuric acid.[18] Since formic acid must have the formula

$$\begin{array}{ccccc} \mathrm{H} & + - & \mathrm{C} & {}^{+}_{+}\,{}^{-}_{-} & \mathrm{O} \\ & & + & & \\ & & - & & \\ & & \mathrm{OH} & & \end{array}$$

the carboxyl group from which it was derived must be triply positive and it must have been attached to the neighboring carbon atom as follows:

$$\begin{array}{ccccc} \mathrm{C} & + - & \mathrm{C} & {}^{+}_{+}\,{}^{-}_{-} & \mathrm{O} \\ & & + & & \\ & & - & & \\ & & \mathrm{OH} & & \end{array}$$

4. The above carboxylated compounds give off carbon monoxide when they are warmed gently with fuming sulfuric acid. Carbon monoxide must have the formula ${}^{+}_{-}\mathrm{C}{}^{+}_{+}\,{}^{-}_{-}\mathrm{O}$. The carbon is identical, electrically, with the carbon in formic acid. Its evolution has a significance identical with that ascribed to formic acid in the preceding paragraph.

A similar decomposition occurs with phosphoric acid.

The results are less reliable, however, because much higher temperatures are required to start the decomposition.

Principle III. Hydrolysis.—Frequent use has been made of the principle of hydrolysis which has been so thoroughly discussed by others[19] that a repetition seems superfluous.

Assumptions.

1. The charges on the carbon atoms are definitely polarized in at least the large majority of organic compounds.

There has been a tendency in recent years to assume that organic compounds are non-polar.[20] An entirely non-polar com-

[18] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726.

[19] Nelson, J. M., Beans, H. T., and Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 1810. Selivanow, *Ber. chem. Ges.*, 1892, xxv, 3517. Stieglitz, J., *Am. Chem. J.*, 1896, xviii, 756. Noyes, W. A., *J. Am. Chem. Soc.*, 1913, xxxv, 769.

[20] For a criticism of this view see Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1914, xxxvi, 209.

pound would be chemically inactive. It may, of course, be true that some or even most of the molecules are really non-polar but such molecules can be of no importance in initiating chemical reactions. *Such inactive non-polar molecules must always be in equilibrium with a certain number of active polar molecules. These active molecules are responsible for the chemical activity of the compound and they are very definitely polar.*[21] The present paper is limited to a discussion of the active polar molecules.

2. The electrical charges hold their places until they are forced to shift because of a definite overwhelming strain. Such shifts are infrequent, belong to classes of compounds rather than to individuals, and must never be assumed to have occurred unless this can be undeniably proved.[22]

References.

It will be necessary to refer frequently to the carbon atoms and to the bonds between the carbon atoms. Instead of placing numbers on the carbon atoms in each of the formulas, which would lead to confusion, the general rule has been adopted of numbering the carbon atoms mentally from *right* to *left* in a horizontal formula and from the top down in a vertical formula. A concrete example will make this clear. Crotonic acid has the structural formula $CH_3-CH=CH-COOH$. The carboxyl carbon is carbon atom 1, and the double bond is the (2–3) bond.

The facts, upon which the electronic formulas are based, can be verified by referring to any large handbook of organic chemistry such as Beilstein's Handbuch der Organischen Verbindungen and Richter's Organic chemistry.

A Consideration of the Formulas.

Propiolic Acid ($H-C\equiv C-COOH$).

When the potassium salt of this acid is heated with water it decomposes smoothly according to the following equation.

[21] Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1448.

[22] Examples of such shifts can be found in the numerous papers by Stieglitz and his collaborators on the electronic interpretation of the Beckmann rearrangement.

$$2\,\mathrm{HC} \equiv \mathrm{C} - \mathrm{COOK} + \mathrm{H_2O} \longrightarrow 2\,\mathrm{C_2H_2} + \mathrm{CO_2} + \mathrm{K_2CO_3}$$

Since the carboxyl group is eliminated as CO_2, carbon atom 1 must be quadruply positive.

When propiolic acid is treated with the halogen acids, only the β-halogenated acrylic acids are formed. The exclusive formation of beta derivatives proves that the triple bond is unsymmetrically polar, the electrons being attached to carbon atom 2. The entire formula for propiolic acid, is, therefore,

$$\mathrm{H} + - \mathrm{C} \begin{smallmatrix}+ & -\\ + & -\\ + & -\end{smallmatrix} \mathrm{C} - + \underset{\substack{+\\ -\\ \mathrm{OH}}}{\mathrm{C}} \begin{smallmatrix}+ & -\\ + & -\end{smallmatrix} \mathrm{O} \tag{1}$$

β-Halogenated Propiolic Acids ($X-C\equiv C-COOH$).

The halogenated propiolic acids decompose readily into $X-C\equiv C-H$ and CO_2. The carboxyl group is, therefore, quadruply positive.

The halogenated propiolic acids react with the halogen acids to give beta derivatives exclusively. Thus β-chloropropiolic acid gives β-dichloroacrylic acid with HCl. This proves that the triple bond is unsymmetrically polar so that the electrons are attached to carbon atom 2. The entire formula for this class of compounds is

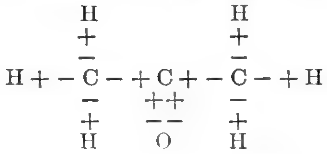
$$\mathrm{X} - + \mathrm{C} \begin{smallmatrix}+ & -\\ + & -\\ + & -\end{smallmatrix} \mathrm{C} - + \underset{\substack{+\\ -\\ \mathrm{OH}}}{\mathrm{C}} \begin{smallmatrix}+ & -\\ + & -\end{smallmatrix} \mathrm{O} \tag{2}$$

The carbon atoms in these acids are identical, electrically, with the carbon atoms in malonic acid.

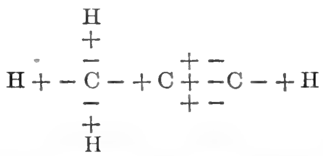
Allylene ($CH_3-C\equiv C-H$).

Allylene is readily absorbed by the halogen acids, the products being $CH_3-CCl_2-CH_3$, $CH_3-CBr_2-CH_3$, and $CH_3-CI_2-CH_3$. In every case the negative halogen attaches itself to the central carbon atom. This unsymmetrical addition of the halogen acids proves that the triple bond is unsymmetrically polar, the electrons being attached to carbon atom 1.

When allylene is heated with dilute sulfuric acid, acetone is formed. Here again the negative oxygen attaches itself to the central carbon atom. Acetone has been proved[23] to have the formula



Both of the end-carbon atoms are quadruply negative. The methyl group in methyl acetylene must, therefore, also be quadruply negative. The entire formula for methyl acetylene is

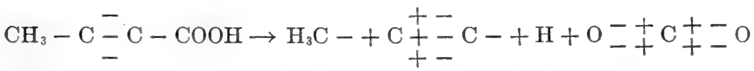


(3)

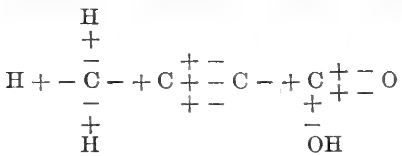
The carbon atoms in allylene are identical, electrically, with the carbon atoms in acetone.

Tetrolic Acid ($CH_3-C\equiv C-COOH$) and Its Relation to Acetoacetic Acid.

When tetrolic acid is heated to 210° it decomposes into allylene and carbon dioxide.



The complete electronic formula for tetrolic acid is, therefore,



(4)

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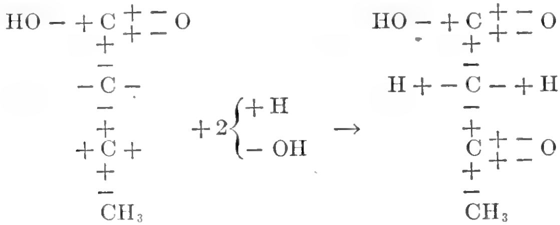
[23] See foot-note 2. This paper also contains the proof of the formulas for acetic, acetoacetic, acetone dicarbonic, and citric acids.

Since the above decomposition occurs at 210° it alone would not suffice to establish the formula for tetrolic acid. That the above formula is actually correct is proved by the following reactions for tetrolic acid.

It combines with the halogen acids to give β-halogenated tetrolic acids.

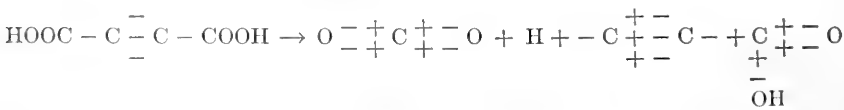
When heated to 105° with a concentrated solution of potassium hydroxide, it decomposes completely with the formation of large quantities of acetone and carbon dioxide and a small amount of acetic acid. This strongly suggests the intermediate formation of acetoacetic acid. This is to be expected from the assigned formula, because the carbon atoms in acetoacetic and in tetrolic acids are electronically identical.



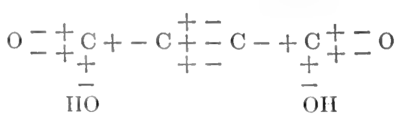
The acetoacetic acid would then give acetone and CO_2 in preponderance, and acetic acid in small amounts as is its habit.

Acetylene Dicarboxylic Acid ($HOOC-C\equiv C-COOH$).

When this acid is warmed with water it decomposes into propiolic acid and carbon dioxide.



Both carboxyl groups are quadruply positive. The formula for acetylene dicarboxylic acid is



(5)

Acrylic Acid ($CH_2 = CH - COOH$).

The recorded reactions for this substance are:

1. It unites with HCl and HI to give β-chloro- and β-iodo-propionic acid, respectively.

2. It gives β-oxypropionic acid, not lactic acid, when heated to 100° with an aqueous solution of sodium hydroxide.

3. When ethyl acrylate is heated with ethyl alcohol containing some sodium ethylate, β-ethoxypropionic acid is formed.

These three entirely one-sided reactions prove that the charges constituting the double bond are polarized so that the electrons are attached to carbon atom 2.

When potassium acrylate is fused with potassium hydroxide, hydrogen, potassium formate, and potassium acetate are formed. This reaction is obviously an hydrolysis at the double bond in which two positive hydrogen atoms are united with the alpha and two negative hydroxyl radicals with the beta carbon atom in exactly the manner that would be expected from the charges assigned to the double bond.

$$CH_2 = CH - COOK + H_2O \rightarrow H_2C = O + CH_3 - COOK$$

The formaldehyde formed at first reacts in the customary manner with the potassium hydroxide to give hydrogen and potassium formate.

$$H_2C = O + KOH \rightarrow H_2 + HCOOK$$

The most important fact from our point of view, is the formation of acetic acid. The formula for this substance has been proved[23] to be

$$\begin{array}{ccccc} & H & & & \\ & + & & & \\ & - & & & \\ H + - & C & - + & C & \begin{smallmatrix}+ & - \\ + & -\end{smallmatrix}\, O \\ & - & & + & \\ & + & & - & \\ & H & & OH & \end{array}$$

It contains a quadruply positive carboxyl group. The carboxyl group in acrylic acid must, therefore, also be quadruply positive. This leads to the following complete formula for acrylic acid.

$$\begin{array}{ccccccccc} & & H & & H & & & & \\ & & + & & + & & & & \\ & & - & & - & & & & \\ H & + - & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & C & - + & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & O \\ & & & & & & + & & \\ & & & & & & - & & \\ & & & & & & OH & & \end{array} \qquad (6)$$

Cinnamic Acid ($C_6H_5-CH = CH-COOH$).

When cinnamic acid is treated with HBr or HI, β-halogenated phenylpropionic acids are formed exclusively. Hypochlorous acid is absorbed so that the negative hydroxyl group attaches itself to the beta carbon atom the product being phenyl α-chloro, β-lactic acid. *The exclusive formation of beta derivatives proves that the double bond* is unsymmetrically polar so that the electrons are attached to the alpha carbon atom.

When cinnamic acid is fused with potassium hydroxide, benzoic acid and acetic acid are the products, the negative OH group being *again*, at this high temperature, attracted *to the beta position.* The formation of acetic acid proves the carboxyl group to be quadruply positive. The formula for cinnamic acid is

$$\begin{array}{ccccccccc} C_6H_5 & - & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & C & - + & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & O \\ & & - & & - & & + & & \\ & & + & & + & & - & & \\ & & H & & H & & OH & & \end{array}$$

The remaining unsolved bond is probably (+ −), because cinnamic acid gives ortho and para derivatives with HNO_3 exactly as is the case with phenol, aniline, and the halogenated benzenes in which the substituting group is surely negative. The entire formula for cinnamic acid would then be

$$\begin{array}{ccccccccc} C_6H_5 & + - & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & C & - + & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & O \\ & & - & & - & & + & & \\ & & + & & + & & - & & \\ & & H & & H & & OH & & \end{array} \qquad (7)$$

It is electronically identical with acrylic acid, a positive C_6H_5 group having replaced the positive hydrogen.

β-Chloro-, Brom-, and Oxyphenylpropionic Acid ($C_6H_5-CHX-CH_2-COOH$).

The formulas for these substances follow as a natural corollary from that of cinnamic acid as can be seen from the following equation.

$$
\begin{array}{ccc}
\begin{array}{c}
C_6H_5\\ + \\ - \\ H+-\;C\;+ \\ + \\ - \\ H+-\;C\;- \\ - \\ + \\ HO-+\;C\;{}^{+\,-}_{+\,-}\;O
\end{array}
& +\left\{\begin{array}{l} -X \\ +H \end{array}\right. \rightarrow &
\begin{array}{c}
C_6H_5\\ + \\ - \\ H+-\;C\;+-X \\ + \\ - \\ H+-\;C\;-+H \\ - \\ + \\ HO-+\;C\;{}^{+\,-}_{+\,-}\;O
\end{array}
\end{array} \qquad (8)
$$

(8) is the formula for β-chloro-, brom-, or oxyphenylpropionic acid.

Crotonic Acid ($CH_3-CH\overset{+}{\underset{-}{=}}CH-COOH$).

Crotonic acid combines with hydrogen iodide to give β-iodobutyric acid, with hydrogen bromide to give β-brombutyric acid and with sodium ethylate to give β-ethoxybutyric ester. In every case the negative radical attaches itself to the beta carbon atom. The double bond is, therefore, unsymmetrically polar, the electrons being attached to the alpha carbon atom.

When crotonic acid is fused with potassium hydroxide it is hydrolyzed at the double bond to give acetaldehyde and acetic acid. The acetaldehyde formed at first is not liberated as such but reacts with KOH in the customary manner to give acetic acid and hydrogen.

$$CH_3-CH\overset{+}{\underset{-}{=}}CH-COOK + KOH \rightarrow CH_3-CHO + CH_3-COOK$$
$$CH_3-CHO + KOH \rightarrow CH_3-COOK + H_2$$

At high temperatures the double bond is still unsymmetrically polar so that the negative OH groups attach themselves to the beta carbon atom. The important fact is the formation of 2 molecules of acetic acid because this proves the polarity of the (1–2) and the (3–4) bond. Acetic acid contains a quadruply positive carboxyl group; so the carboxyl group in crotonic acid must also be quadruply positive. The second molecule of acetic acid, coming as it does from the acetaldehyde formed at first, proves that the (3–4) bond must have an electrical polarity like that of acetaldehyde; namely, $(C-+C)$. The entire formula for crotonic acid is

$$\begin{array}{cccccccccc}
 & H & & H & & H & & & & \\
 & + & & + & & + & & & & \\
 & - & & - & & - & & & & \\
H+- & C & -+ & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & C & -+ & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & O \\
 & - & & & & & & + & & \\
 & + & & & & & & - & & \\
 & H & & & & & & OH & &
\end{array} \qquad (9)$$

Crotonic acid contains the acrylic acid nucleus.

β-Oxy- and β-Halogenated Butyric Acids $(CH_3-CHX-CH_2-COOH)$.

The facts that are needed for the proof of these formulas have already been given under crotonic acid. They will be repeated here for the sake of clearness.

When crotonic acid is heated with an aqueous solution of sodium hydroxide, β-oxybutyric acid is formed.

When crotonic acid is heated with hydrogen bromide or iodide, β-brom- and β-iodobutyric acids are formed, respectively. Electronically these reactions proceed as follows.

$$\begin{array}{cccc}
 & CH_3 & & \\
 & - & & \\
 & + & & \\
H+- & C & + & \\
 & + & & \\
 & - & & \\
H+- & C & - & \\
 & - & & \\
 & + & & \\
HO-+ & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & O
\end{array}
\quad + \begin{cases} -X \\ +H \end{cases} \quad \longrightarrow \quad
\begin{array}{cccc}
 & CH_3 & & \\
 & - & & \\
 & + & & \\
H+- & C & +- & X \\
 & + & & \\
 & - & & \\
H+- & C & -+ & H \\
 & - & & \\
 & + & & \\
HO-+ & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & O
\end{array} \qquad (10)$$

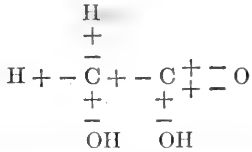
Formula (10) is that of β-oxy, β-ethoxy, and β-halogenated butyric acids. The carbon atoms are electrically identical with the carbon atoms in crotonic acid. It is not surprising, then, to find that they readily lose HX to give crotonic acid.

Glycollic Acid $(CH_2OH-COOH)$.

When glycollic acid is warmed with concentrated sulfuric acid it decomposes into carbon monoxide, water, and trioxymethylene.

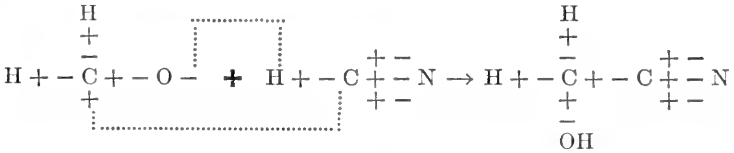
$$3\ CH_2OH - COOH \longrightarrow 3CO + 3H_2O + (CH_2O)_3$$

In this case the carboxyl group is split off as carbon monoxide and the formaldehyde formed at first polymerizes to trioxymethylene. The carboxyl group must, therefore, be triply positive. The complete formula for glycollic acid is



(11)

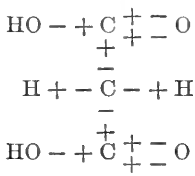
This formula is substantiated by the preparation of glycollic acid from formaldehyde and prussic acid.



The nitrile then gives the acid on hydrolysis which can produce no change in the electrical conditions of the molecule.

Malonic Acid ($COOH-CH_2-COOH$).

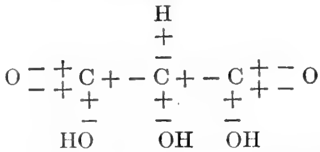
When the acid is heated above its melting point, 132°, it decomposes smoothly into CO_2 and acetic acid. The evolution of CO_2 proves that at least *one* of the carboxyl groups is quadruply positive. Acetic acid has been proved to contain a positive carboxyl group; hence the second carboxyl group in malonic acid must also be quadruply positive. The entire formula for this substance is, therefore,



(12)

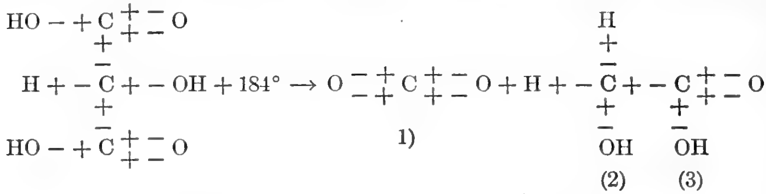
Tartronic Acid ($HOOC-CHOH-COOH$).

When tartronic acid is heated above its melting point, 184°, it decomposes smoothly into carbon dioxide and a polymer of glycollic acid. The evolution of CO_2 proves that *one* of the carboxyl groups is quadruply positive. Glycollic acid has been proved to have a negative carboxyl group. The formula for tartronic acid must then be



(13)

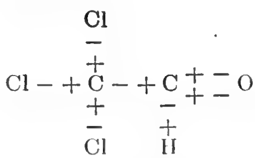
The decomposition can be represented electronically as follows



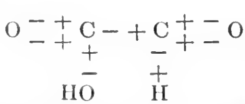
Mesoxalic Acid (HOOC—CO—COOH).

When this acid is heated above its melting point it decomposes into carbon dioxide and glyoxylic acid. The evolution of CO_2 proves that one of the carboxyl groups is quadruply positive. The formula for glyoxylic acid cannot be proved from the recorded reactions of that substance; but the following indirect proof seems rather convincing.

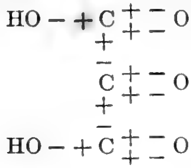
The formula for chloral must be



because it gives chloroform and sodium formate when treated with aqueous alkalies. If it were possible to substitute OH groups for the three negative halogen atoms without separating the carbon atoms, glyoxylic acid would be formed. The formula for glyoxylic acid should then be



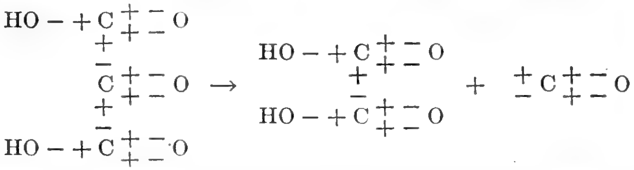
From this one would conclude that one of the carboxyl groups in mesoxalic acid was triply positive and that the entire formula for this substance is



(14)

One assumption has been made; namely, that the substitution of negative oxygen for negative chlorine in chloral has produced no change in the polarity of the charges binding the carbon atoms together.

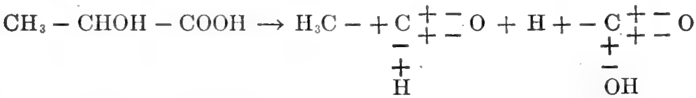
That we were justified in making the above assumption, and that the formula assigned to mesoxalic acid is really correct is proved in a striking manner by the fact that the acid decomposes on boiling with water into carbon monoxide and oxalic acid. Electronically this reaction would be represented as follows.



The carbon monoxide might arise from either the central or the triply positive end-carbon atom. In any case *it would be impossible to obtain CO and oxalic acid from a molecule having any but the above electronic structure.*

Lactic Acid ($CH_3-CHOH-COOH$).

When lactic acid is treated at 60° with fuming sulfuric acid, the carboxyl group is eliminated as carbon monoxide. A similar decomposition occurs when lactic acid is heated to 130° with dilute sulfuric acid, the products being acetaldehyde and formic acid.



(3) (2) (1)

From the above equation the formula for lactic acid is easily seen to be

$$\begin{array}{ccccccc}
 & \mathrm{H} & & \mathrm{H} & & & \\
 & + & & + & & & \\
 & - & & - & & & \\
\mathrm{H}+- & \mathrm{C} & -+ & \mathrm{C} & +- & \mathrm{C} & \substack{+\\+}\substack{-\\-}\mathrm{O} \\
 & - & & + & & + & \\
 & + & & - & & - & \\
 & \mathrm{H} & & \mathrm{OH} & & \mathrm{OH} &
\end{array} \qquad (15)$$

β-Oxypropionic Acid (CH_2OH-CH_2-COOH).

When acrylic acid is heated to 100° with an aqueous solution of sodium hydroxide, β-oxypropionic acid is formed. Electronically, this reaction can be written as follows.

$$\begin{array}{ccccccccc}
 & \mathrm{H} & & & & & & \mathrm{H} & \\
 & + & & & & & & + & \\
 & - & & & & & & - & \\
\mathrm{H}+- & \mathrm{C} & + & & -\mathrm{OH} & & \mathrm{H}+- & \mathrm{C} & +-\mathrm{OH} \\
 & + & & & & & & + & \\
 & - & & & & \rightleftarrows & & - & \\
\mathrm{H}+- & \mathrm{C} & - & + & +\mathrm{H} & & \mathrm{H}+- & \mathrm{C} & -+\mathrm{H} \\
 & - & & & & & & - & \\
 & + & & & & & & + & \\
\mathrm{HO}-+ & \mathrm{C} & \substack{+\\+}\substack{-\\-}\mathrm{O} & & & & \mathrm{HO}-+ & \mathrm{C} & \substack{+\\+}\substack{-\\-}\mathrm{O}
\end{array} \qquad (16)$$

The carbon atoms in β-oxypropionic acid are identical, electronically, with those in acrylic acid. It is not surprising to find, then that β-oxypropionic acid readily loses water to give acrylic acid. To obtain acrylic acid from *lactic acid,* a far reaching electronic rearrangement is necessary and one would expect that to bring about this transformation, a more drastic treatment would be required. This is the case. The difference in ease of activity between the α- and β-oxy (and halogenated) derivatives is readily explained, in this manner by the electronic formulas.

Pyruvic Acid $(CH_3-CO-COOH)$.

When pyruvic acid is heated to 150° with dilute sulfuric acid it is hydrolyzed, the products being acetaldehyde and carbon dioxide.

$$\begin{array}{lcccc}
CH_3-CO-COOH+H_2O \longrightarrow & H_3C-+ & \mathrm{C} & \substack{+\\+}\substack{-\\-}\mathrm{O} & +\ \mathrm{O}\substack{-\\-}\substack{+\\+}\mathrm{C}\substack{+\\+}\substack{-\\-}\mathrm{O}+H_2O \\
 & & - & & \\
 & & + & & \\
 & & \mathrm{H} & & \\
 & (3) & (2) & & (1)
\end{array}$$

From the above partially electronic equation the entire formula for pyruvic acid is readily seen to be

$$
\begin{array}{ccccccc}
 & & \mathrm{H} & & & & \\
 & & + & & & & \\
 & & - & & & & \\
\mathrm{H} & + - & \mathrm{C} & - + & \mathrm{C} & - + & \mathrm{C}\overset{+}{\underset{+}{}} \overset{-}{\underset{-}{}}\mathrm{O} \\
 & & - & & ++ & & + \\
 & & + & & -- & & - \\
 & & \mathrm{H} & & \mathrm{O} & & \mathrm{OH}
\end{array}
\qquad (17)
$$

Both pyruvic and oxalic acids contain the electrical dyad $\left(\begin{smallmatrix}+\\+\\+\end{smallmatrix} C - + C \begin{smallmatrix}+\\+\\+\end{smallmatrix}\right)$. They are similarly hydrolyzed by sulfuric acid.

Malic Acid ($HOOC-CH_2-CHOH-COOH$).

When malic acid is heated with dilute sulfuric acid, it decomposes into CO_2, formic acid, and acetaldehyde. When malic acid is warmed with fuming sulfuric acid it decomposes quantitatively according to the following equation.[24]

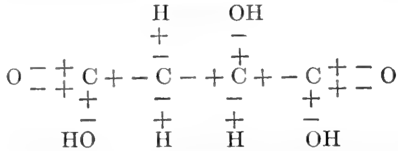
$$
\begin{array}{lccc}
HOOC-CH_2-CHOH-COOH \rightarrow & HOOC-CH_2-CHO & (A) & + \; HCOOH \\
 & \downarrow & & \downarrow \\
 & C_5H_4O_2-COOH & (B) & CO + H_2O
\end{array}
$$

Compound (B) is a polymer of (A). When (B) is boiled with dilute sulfuric acid it decomposes into CO_2 and acetaldehyde. Although compound (A) has never been isolated—it polymerizes too readily—its formation as an intermediate product in the decomposition of malic acid by sulfuric acid is proved conclusively by the character of the condensation products formed when malic acid, sulfuric acid, and phenols are heated together.

This reaction proves the entire formula for malic acid. Since CO is evolved—or formic acid with dilute sulfuric acid—*one* of the carboxyl groups must be negative. The negative carboxyl group is attached to carbon atom 1 (see the above equation). The evolution of CO_2 from the polymer of $HOOC-CH_2-CHO$ proves that the other carboxyl group is quadruply positive. The other product is acetaldehyde, a compound of known electronic constitution (CH_3-+CHO).

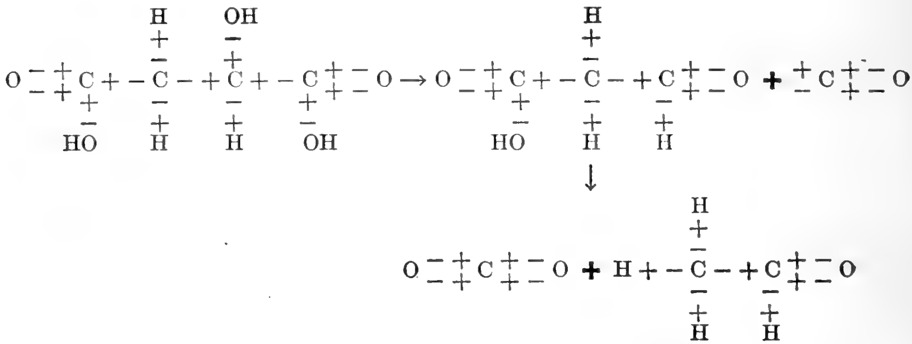
[24] von Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, 1884, xvii, 929, 1649.

The entire formula for malic acid is



(18)

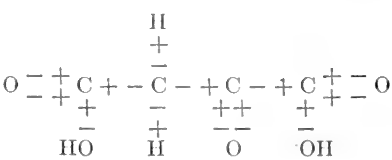
This proof is rendered clear by expressing the decomposition electronically.



Oxalacetic Acid (*HOOC—CH₂—CO—COOH*).

The formula for this substance is clearly proved by the following decompositions of its diethyl ester. When boiled with alkalies it is hydrolyzed so that the products are oxalic acid, acetic acid, and alcohol. When boiled with dilute acids it is hydrolyzed so that carbon dioxide and pyruvic acid are formed. When heated under ordinary pressures it decomposes into carbon monoxide and malonic ethyl ester.

Only one formula can be written that readily explains all of these decompositions; namely,



(19)

As can be seen, an hydrolysis at bond (2–3) would give acetic and oxalic acids; at bond (3–4) would give carbon dioxide and pyruvic acid. The loss of carbon atom 2 would give carbon monoxide and malonic acid.

Tartaric Acid (HOOC–CHOH–CHOH–COOH).

Tartaric acid reacts readily with concentrated sulfuric acid or phosphoric acid. The several observers seem to have obtained different products when operating under slightly different conditions. Thus Vangel[25] found that when tartaric acid is heated to 150° with phosphoric acid, equal volumes of CO_2 and CO were formed. He did not examine the non-gaseous residue, nor did he measure the exact quantity of gas obtained per unit weight of tartaric acid. Bouchardat[26] found that when tartaric acid is warmed to 40–50° with fuming sulfuric acid containing 80 per cent of SO_3, a gas is formed that is composed of 4 parts of CO and 1 part of SO_2, and which contains from 2 to 4 per cent of carbon dioxide. He states, moreover, that the carbon dioxide and SO_2 appeared toward the *end* of the reaction. An examination of the non-gaseous residue revealed the presence of some racemic tartaric acid and small amounts of glycollic and pyruvic acids. He gives no data by means of which one could calculate how many of the carbon atoms of tartaric acid were evolved as carbon monoxide. Since these data were necessary to establish the electronic formula for tartaric acid, the following experiment was carried out.

Tartaric acid—1.5000 gm., M.P. 170°—was mixed in a 50 cc. round bottomed flask with 25 cc. of fuming sulfuric acid containing 18 per cent of SO_3. The flask was connected to a 1,000 cc. narrow mouthed precision cylinder that had been inverted and arranged so that the evolved gases could be collected by displacement of water. The flask was heated to 65°. A gas was slowly and steadily evolved that was pure carbon monoxide at first. Not a trace of either SO_2 or CO_2 was present until the gas volume, corrected for temperature, pressure, and aqueous tension was 445 cc. At this point the evolution of gas becomes livelier.

[25] Vangel, B., *Ber. chem. Ges.*, 1880, xiii, 356.
[26] Bouchardat, M. G., *Bull. Soc. chim.*, 1880, xxxiv, series 2, 495.

Considerable SO_2 was present as evidenced by the white fumes that formed when the gas came in contact with the wet walls of the cylinder. The reaction was practically over when about 950 cc. of gas had been collected. The volume shrunk to 840 cc. on agitation with water. This removed all of the SO_2 and probably also some of the CO_2. A subsequent agitation with aqueous sodium hydroxide gave a final volume of 800 cc. of gas that proved to be pure carbon monoxide.

The sulfuric acid was colored very pale yellow after the reaction was over so that no charring had occurred.

An examination of this reaction shows the following. Carbon monoxide (445 cc.) was evolved from 1.5 gm. of tartaric acid before either SO_2 or CO_2 were formed. This amount of CO was liberated by the primary action of the sulfuric acid before an oxidation reaction had occurred. This quantity of tartaric acid should give about 225 cc. of CO per carbon atom or 900 cc. if all of the carbon atoms had been converted into CO. The 445 cc. actually obtained represent two carbon atoms, half of the tartaric acid molecule. Since two of the four carbon atoms were evolved as CO *before an oxidation reaction had occurred*, it is fairly safe to conclude that *both* of the *carboxyl groups* were eliminated as CO according to the following equation

$$(CHOH)_2\,(COOH)_2 \;\longrightarrow\; 2CO + CHO - CHO + 2H_2O$$

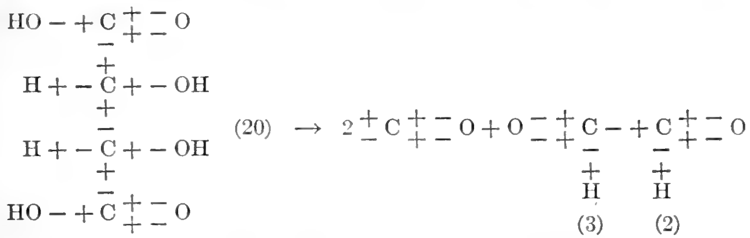
Both of the carboxyl groups must, therefore, have been triply positive. The assumption that glyoxal is an intermediate product in the decomposition of tartaric acid by fuming sulfuric acid is in perfect agreement with the observed fact that all of the carbon atoms in tartaric acid are finally evolved as CO. A primary oxidation of glyoxal would yield glyoxylic acid which would then be converted largely into CO and to a small extent into oxalic acid, CO, and CO_2.

$$1.\begin{cases} CHO - CHO + H_2SO_4 & \longrightarrow\; CHO - COOH + SO_2 + H_2O \\ CHO - COOH & \longrightarrow\; HCOOH \quad + CO \\ HCOOH & \longrightarrow\; CO \quad + H_2O \end{cases}$$

$$2.\begin{cases} CHO - COOH + H_2SO_4 & \longrightarrow\; COOH - COOH + SO_2 + H_2O \\ COOH - COOH & \longrightarrow\; CO \quad + CO_2 \end{cases}$$

The evolution of so much CO and so little CO_2 could not be explained at all if the assumption were made that even one of the carboxyl groups in tartaric acid was quadruply positive.

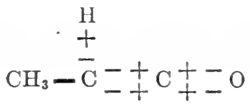
The complete electronic formula for tartaric acid and its primary decomposition into CO, glyoxal, and water can be represented as follows.



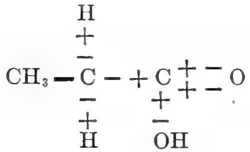
(20)

Propionic Acid (CH_3-CH_2-COOH).

That the carboxyl group in propionic acid is quadruply positive is proved by the absorption reactions of methyl ketene, $CH_3-CH=C=O$. Methyl ketene unites with water to give propionic acid, with alcohol to give ethyl propionate, and with ammonia to give propionamide. In every case the negative radical attaches itself to carbon atom 1. This proves that the double bond is unsymmetrically polar; that the partial formula for methyl ketene is

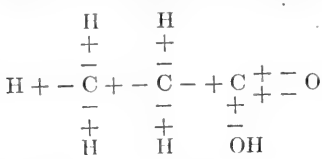
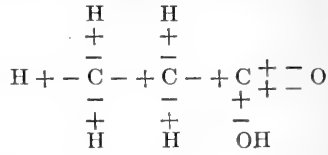


and that propionic acid has the partial formula



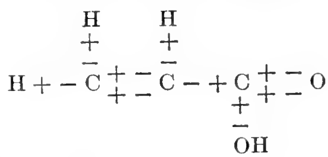
The polarity of the remaining unsolved bond cannot be determined directly. The following partially speculative proofs seem convincing to the authors.

Just two formulas are possible, namely,

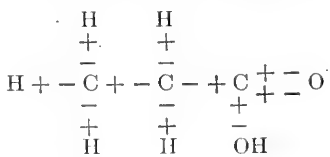
(1)  (2) 

It is, of course, possible that both of these formulas are correct and that the two electrons between carbon atoms 2 and 3 are vibrating so that they are attached first to one and then to the other carbon atom (or they may hold an electron in common). Although the possibility of such an electrometric equilibrium is undeniable, we have no right to assume its existence without proof. Most of the simple compounds examined so far have shown a rigid polarity. In the absence of proof to the contrary we will assume that the same rigidity exists in this compound. There are two proofs that formula (1) is representative of the electrical conditions existing in the molecule of propionic acid.

1. Acrylic acid has been proved to have the formula



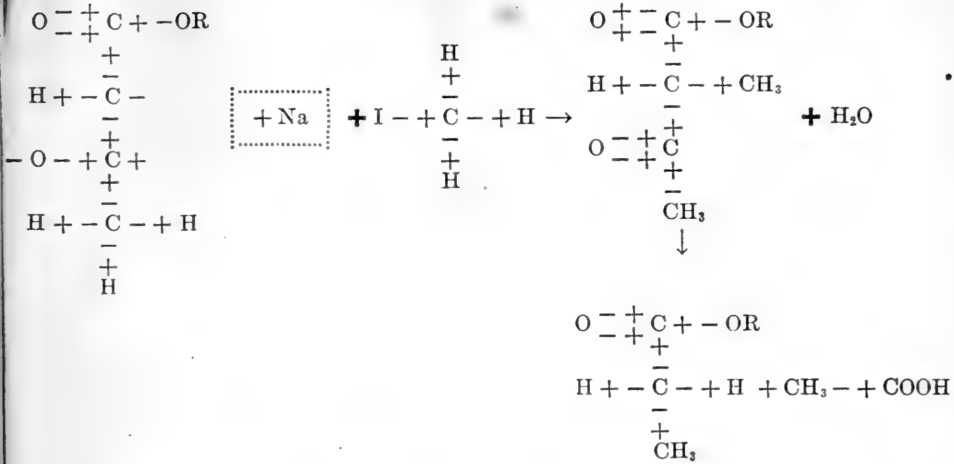
By reduction acrylic acid gives propionic acid. The reduction consists in the addition of two electrons to the beta carbon atom. The formula for propionic acid should then be



(21)

One assumption must be made; namely, that an electronic shift does not take place *within the molecule after the electrons and the hydrogen atoms have been added.*

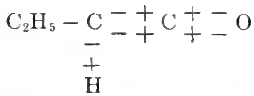
2. Propionic acid can be prepared from the sodium compound of acetoacetic or malonic ester and methyl iodide. The reactions can be represented electronically as follows.



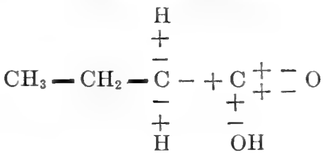
This formula is also obtained by the similar reaction of methyl iodide with the sodium compound of malonic ester and is identical with formula (1).

Butyric Acid ($CH_3-CH_2-CH_2-COOH$).

The carboxyl group in butyric acid is quadruply positive. This can be proved from the absorption reactions of ethyl ketene ($CH_3-CH_2-CH\overset{-}{\underset{-}{}}C\overset{-}{\underset{-}{}}O$). Ethyl ketene absorbs water, alcohol, and aniline so that the negative OH, OC_2H_5, and $NH-C_6H_5$ groups attach themselves to carbon atom 1, the products being butyric acid, ethyl butyrate, and butyric acid anilide. These entirely one-sided reactions prove that the double bond in ethyl ketene is unsymmetrically polar so that the electrons are attached to carbon atom 2. The partial formula for ethyl ketene is, therefore,



The partial formula for butyric acid must then be



The remaining bonds can be solved only by speculation as follows. Crotonic acid has been proved to have the formula

$$\begin{array}{ccccccc}
H_3C\,- & +C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & C & -\,+ & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix}\,O \\
 & - & & - & & + & \\
 & + & & + & & - & \\
 & H & & H & & OH &
\end{array}$$

By reduction, crotonic acid gives butyric acid. The reduction consists in the addition of two electrons to the beta carbon atom. The formula for butyric acid should then be

$$\begin{array}{ccccccccc}
 & H & & H & & H & & & \\
 & + & & + & & + & & & \\
 & - & & - & & - & & & \\
H\,+\,- & C & -\,+ & C & +\,- & C & -\,+ & C & \begin{smallmatrix}+&-\\+&-\end{smallmatrix}\,O \\
 & - & & - & & - & & + & \\
 & + & & + & & + & & - & \\
 & H & & H & & H & & OH &
\end{array} \tag{22}$$

Although this must certainly be the formula at the moment of reduction, the objection might be raised that the electrical charges could shift after the hydrogen atoms and the electrons had been added. To assume that such a shift did occur would be unreasonable unless the molecule so formed contained electrical dyads of uncertain stability. On the other hand, if the certain stability of every dyad in the molecule could be proved, the above formula should be considered correct until some definite proof to the contrary was found. The undoubted stability of every dyad in this formula is proved by considering the electronic skeleton of the acrylic acid molecule.

$$\begin{array}{ccccc}
 & - & - & + & \\
+ & C\,+\,- & C\,-\,+ & C & + \\
 & - & - & + &
\end{array}
\qquad
\begin{array}{cccccc}
 & - & - & - & + & \\
- & C\,-\,+ & C\,+\,- & C\,-\,+ & C & + \\
 & - & - & - & + &
\end{array}$$

(Acrylic acid.) (Butyric acid.)

The dyad $\begin{array}{c} \\ +\overset{-}{\underset{-}{C}}+-\overset{-}{\underset{-}{C}}- \\ \end{array}$ appears once in the electrically very stable acrylic acid and twice in the assigned formula for butyric acid. The formula assigned to butyric acid is, therefore, not only possible but extremely probable.

Phenylpropionic Acid ($C_6H_5-CH_2-CH_2-COOH$).

The method used in proving the formula for phenylpropionic acid is almost identical with that just described for propionic

acid. A detailed proof is withheld to avoid unnecessary repetition. The formula is

$$\begin{array}{ccccccc} & & H & & H & & \\ & & + & & + & & \\ & & - & & - & & \\ C_6H_5 & +- & C & +- & C & -+ & C \overset{+-}{\underset{+-}{=}} O \\ & & - & & - & & + \\ & & + & & + & & - \\ & & H & & H & & OH \end{array} \qquad (23)$$

The charge on the benzene ring is given as positive because phenylpropionic acid gives ortho and para derivatives just as is the case with all of the negatively mono-substituted derivatives of benzene. Phenylpropionic acid is electronically identical with propionic acid, a positive hydrogen atom having been replaced by a positive phenyl group.

Two New Electronic Principles.

Principle IV.—The polarity of the double bond in an unsaturated aliphatic compound is determined by the electrical charge on the first substituting group.

An examination of the formulas just presented has led us to conclude that the polarity of the double bond in an unsaturated aliphatic compound is determined by the electrical charge on the first substituting group which we will call the directing group.

There are two sharply defined types of unsaturated compounds, those belonging to the *vinyl type*

$$H_2C \overset{-+}{\underset{-+}{=}} \underset{H}{C} +- V$$

and those belonging to the *acrylic acid type*

$$H_2C \overset{+-}{\underset{+-}{=}} \underset{H}{C} -+ A$$

When the first substituent is negative the compound belongs to the vinyl type. Such a compound will always give alpha derivatives by the absorption of a polar compound like HCl. When the first substituent is positive the compound belongs to the acrylic acid type; it gives only beta derivatives by the absorption of polar compounds like HCl.

Beside these two simple types, one very stable and common mixed type is possible; namely, $V -+ C \overset{+-}{\underset{+-}{=}} C -+ A$. A com-

pound belonging to this type is both a vinyl and an acrylic acid derivative. Such compounds are electrically very stable because their polarity is fixed by two groups both of which are striving to produce the same electrical configuration.

Of the compounds discussed so far, allylene belongs to the vinyl type, propiolic acid to the acrylic acid type, and crotonic, cinnamic, tetrolic, and the β-halogenated propiolic acids belong to the mixed type. The chemical properties of many unsaturated compounds are too incompletely known to make it possible to prove their electronic formulas completely. In such cases Principle IV is frequently of service because it gives us a means of writing a fairly authentic formula on the basis of a few absorption reactions. Examples of such usage will be given presently.

Principle V.—A carbon atom that is attached to an oxygen atom will become at least doubly positive, when this is at all possible.

Glycollic acid is the simplest example of the truth of this principle. The electronic formula for acetic acid is

$$\begin{array}{ccccc} & \mathrm{H} & & & \\ & + & & & \\ & - & & & \\ \mathrm{H}+- & \mathrm{C} & -+ & \mathrm{C} & \begin{smallmatrix}+ & - \\ + & -\end{smallmatrix}\,\mathrm{O} \\ & - & & + & \\ & + & & - & \\ & \mathrm{H} & & \mathrm{OH} & \end{array}$$

When one of the paraffin hydrogen atoms is replaced by an OH group, the compound formed, glycollic acid, has the formula

$$\begin{array}{ccccc} & \mathrm{H} & & & \\ & + & & & \\ & - & & & \\ \mathrm{H}+- & \mathrm{C} & +- & \mathrm{C} & \begin{smallmatrix}+ & - \\ + & -\end{smallmatrix}\,\mathrm{O} \\ & + & & + & \\ & - & & - & \\ & \mathrm{OH} & & \mathrm{OH} & \end{array}$$

The introduction of one OH group has caused carbon atom 2 to lose *four* electrons. From this it would seem that a triply negative carbon atom is electrically unstable when it is combined with oxygen. The triply negative carbon atom will, when this is possible, lose two electrons to a neighboring carbon atom. Of the compounds discussed so far, citric, tartronic, lactic, malic, and tartaric acids can be cited as illustrations of the truth of this principle. All of these compounds are α-hydroxy-acids and

they all contain a negative carboxyl group. In all of these cases, the alpha carbon atom, which is united with the OH group, is at least doubly positive.

The real value of Principles IV and V lies in the fact that they give us a means of accurately predicting the behavior of certain classes of organic compounds and of establishing the formulas for some compounds whose chemical properties are too incompletely known to make a complete proof of their electronic formulas possible. We will now consider a few such examples.

Propylene $(CH_2{=}CH-CH_3)$.

In all of its absorption reactions, propylene gives only isopropyl derivatives. Thus HCl, HBr, and HI give isopropyl chloride, bromide, and iodide respectively. Sulfuric acid gives isopropyl sulfate which by hydrolysis gives isopropyl alcohol. This proves that the electrical charges constituting the double bond are unsymmetrically polar, the electrons being attached to carbon atom 3.

$$\begin{array}{cccccc} H\,+\,- & C & \begin{smallmatrix}-&+\\-&+\end{smallmatrix} & C & \textbf{—} & CH_3 \\ & - & & - & & \\ & + & & + & & \\ & H & & H & & \end{array}$$

The polarity of the remaining unsolved bond cannot be determined directly. That the CH_3 group carries a negative charge is rendered highly probable by the following line of reasoning.

The polarity of the double bond in propylene is like that in the vinyl compounds. Propylene must, then, be a vinyl derivative; hence by Principle IV, the methyl group is negative. The complete formula for propylene is

$$\begin{array}{cccccc} H\,+\,- & C & \begin{smallmatrix}-&+\\-&+\end{smallmatrix} & C & +\,- & CH_3 \\ & - & & - & & \\ & + & & + & & \\ & H & & H & & \end{array} \qquad (24)$$

That an unopposed methyl group normally carries a negative charge is clearly shown by an examination of some aromatic and aliphatic compounds. Toluene gives ortho and para derivatives almost exclusively just as is the case with phenol and chlorobenzene. The methyl group, like the OH and Cl radicals, functions negatively. Negative methyl groups have also been encountered

in acetic acid, acetone, acetoacetic acid, tetrolic acid, allylene, acetone dicarbonic acid, and citric acid. Propionic acid is the only compound that we have encountered up to date that *may* contain a positive methyl group.

Allyl Alcohol ($CH_2{=}H-CH_2OH$).

When allyl alcohol is heated with a saturated solution of potassium acid sulfite, $CH_2SO_3H-CH_2-CH_2OH$ is formed, the negative SO_3H group going to the beta carbon atom.

When allyl alcohol is treated with hypochlorous acid, $CH_2OH-CH_2Cl-CH_2OH$ is formed, the negative OH group attaching itself to the beta carbon atom.

This proves that the double bond in allyl alcohol is unsymmetrically polar so that the electrons are attached to carbon atom 2. The charge on the CH_2OH group cannot be determined directly; but there is good evidence that it is positive and that the entire formula for allyl alcohol is

$$\begin{array}{ccccccc} & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & \\ & + & & + & & + & \\ & - & & - & & - & \\ \mathrm{H}+- & \mathrm{C} & \genfrac{}{}{0pt}{}{+\,-}{+\,-} & \mathrm{C} & -\,+ & \mathrm{C} & +\,\mathrm{OH} \\ & & & & & - & \\ & & & & & + & \\ & & & & & \mathrm{H} & \end{array} \qquad (25)$$

The indirect proof of this statement is as follows.

1. Allyl alcohol gives only beta derivatives when it absorbs electrically polar compounds. In this respect it behaves exactly like acrylic acid, and it belongs, therefore, to the acrylic acid type, *not* to the vinyl type. The CH_2OH group should, therefore, be positive like the carboxyl group in acrylic acid (Principle IV).

2. By Principle V, a carbon atom that is attached to oxygen will become at least doubly positive. The oxymethyl group in allyl alcohol should, therefore, be positive.

3. To assume that the oxymethyl group was negative would be unreasonable, because if that were true, the polarity of the double bond in allyl alcohol and in propylene should be identical. We can see no reason why an added oxygen atom should change the *remote* electrical charges on the molecule if it were incapable of changing the charge on the carbon atom to which it is attached.

Allyl Chloride $(CH_2\overline{\underline{\ }}H-CH_2Cl)$.

Allyl chloride combines with hydrogen chloride at 100° to give propylene chloride, $CH_3-CHCl-CH_2Cl$. Concentrated sulfuric acid is readily absorbed by allyl chloride at room temperatures. When the resulting acid sulfate is treated with water, the product is $CH_3-CHOH-CH_2Cl$. The negative group attaches itself to carbon atom 2 in each case. These facts prove that for temper ıture ranges from 20 to 100° the charges constituting the double bond are unsymmetrically polar, *the electrons being attached to the beta carbon atom.* The partial formula for allyl chloride is

$$\begin{array}{ccccc} & & & H & \\ & & & + & \\ & & & - & \\ H+- & C & {}^{-+}_{-+} & C & -CH_2Cl \\ & - & & & \\ & + & & & \\ & H & & & \end{array}$$

The charge on the CH_2Cl group cannot be determined directly; but there is good evidence that it is negative and that the complete formula for allyl chloride is

$$\begin{array}{ccccccc} & & & H & & H & \\ & & & + & & + & \\ & & & - & & - & \\ H+- & C & {}^{-+}_{-+} & C & +- & C & +-Cl \\ & - & & & & - & \\ & + & & & & + & \\ & H & & & & H & \end{array} \qquad (26)$$

The absorption reactions of allyl chloride are identical with those of propylene and the vinyl derivatives but the reverse of those of acrylic acid and allyl alcohol. Allyl chloride is, therefore, a vinyl derivative; the charge on the CH_2Cl group is negative. The CH_2OH group has just been shown to be *positive.* Although the negative oxygen atom will, when possible, repel two electrons from the carbon atom to which it is attached, this seems not to be the case with the negative chlorine atom. Stated differently, when a carbon atom is attached to an oxygen atom the carbon atom will be charged $^{+}_{-}C^{+}_{-}$ in preference to $^{-}_{-}C^{+}_{-}$, the latter form being rarely capable of existence. *A carbon atom that is attached to chlorine is perfectly stable when it is charged*

$\bar{\underline{}}C\overset{+}{\underline{}}$. This would indicate that triply negative carbon is not *inherently* unstable; it is the attached oxygen atom that renders it so.

The negative charge assigned to the CH_2Cl group is in perfect agreement with its directing force as manifested in the aromatic series. Benzyl chloride gives ortho and para derivatives on halogenation and nitration just as is the case with all of the negatively mono-substituted benzene derivatives.

It might not be out of place at this point to call attention to the need of certain facts that would help greatly to establish the electronic formulas of some biologically very important compounds.

1. Since the CH_2OH group in allyl alcohol is surely positive, it ought also to be positive in benzyl alcohol. Then benzyl alcohol or any of the ethers derived from it should direct the second incoming substituent predominantly to the meta position.

2. It is very important for protein chemistry that we know how the NH_2 group affects the carbon atom to which it is attached. Triply negative carbon has been shown to be stable when attached to chlorine and unstable when attached to oxygen. Although $CH_2OH+-COOH$ is surely the formula for glycollic acid, $CH_2Cl-+COOH$ is probably, therefore, the formula for monochloroacetic acid. What is the formula for aminoacetic acid? This will be hard to prove directly because glycine is an extremely stable compound; but the determination of the following facts would render an indirect proof possible. Does allyl amine give alpha or beta derivatives when it is treated with polar compounds? Does benzyl amine give ortho and para or meta derivatives? From these facts it would be possible to conclude whether the NH_2 group repelled electrons from the carbon atom to which it is attached as is the case with the OH group, or whether triply negative carbon is stable when attached to the NH_2 group as it is when attached to chlorine.

3. Does oxygen repel electrons from the carbon atom to which it is attached *because of its strongly negative character?* This could be determined by examining the properties of certain fluorine derivatives. Since fluorine is more decidedly negative than oxygen, it should be able to repel electrons from the carbon atom to which it is attached at least as certainly as oxygen *if this repulsion was occasioned by the negative character of the fluorine atom.* The oxy-

gen atom may prove to be unique in this respect, which might throw some light upon the intra-atomic structure of this atom. The absorption reactions of allyl fluoride and the behavior of benzyl fluoride on nitration, etc., would settle this point.

Acrolein ($CH_2 \overset{-}{\underset{-}{}} CH - CHO$).

When acrolein is treated at low temperatures with hydrogen chloride or hydrogen bromide, β-chloro- and β- brompropionaldehyde are formed respectively. This proves that the electrical charges constituting the double bond are unsymmetrically polar, the electrons being attached to carbon atom 2. The partial formula for acrolein is

$$\begin{array}{cccccccc} & & & \mathrm{H} & & & & \\ & & & + & & & & \\ & & & - & & & & \\ \mathrm{H}+- & \mathrm{C} & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & \mathrm{C} & \mathbf{-} & \mathrm{C} & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & \mathrm{O} \\ & - & & & & - & & \\ & + & & & & + & & \\ & \mathrm{H} & & & & \mathrm{H} & & \end{array}$$

The charge on the aldehyde group cannot be determined directly; but there is good evidence that it is positive and that the complete formula for acrolein is

$$\begin{array}{cccccccc} \mathrm{H}+- & \mathrm{C} & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & \mathrm{C} & -+ & \mathrm{C} & \begin{smallmatrix}+&-\\+&-\end{smallmatrix} & \mathrm{O} \\ & - & & - & & - & & \\ & + & & + & & + & & \\ & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \end{array} \qquad (27)$$

1. The absorption reactions of acrolein are identical with those of acrylic acid and allyl alcohol; but the reverse of those of the vinyl derivatives. Acrolein, therefore, belongs to the acrylic acid type; the charge on the aldehyde group is positive (Principle IV).

2. Since the charge on the carboxyl group of acrylic acid has been proved to be positive and that on the oxymethyl group of allyl alcohol was proved to be most probably positive, it would be strange indeed if the aldehyde group, which stands between the CH_2OH and the COOH groups in degree of oxidation, should be negative.

3. Aldehyde groups are usually if not invariably positive. In glyoxylic acid, for example, where an aldehyde and a carboxyl group vie for the electron, it is the aldehyde group that is positive. In the aromatic series the aldehyde group directs the second incoming substituent predominantly to the meta position as is the case with nitrobenzene.

STUDIES ON PROTEINOGENOUS AMINES.

XIV. A MICROCHEMICAL COLORIMETRIC METHOD FOR ESTIMATING TYROSINE, TYRAMINE, AND OTHER PHENOLS.

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INTRODUCTION.

The discovery of the diazonium salts by Griess in 1866[1] opened up a most fruitful field for scientific investigation by giving the investigator a class of chemical compounds that are highly susceptible to a large variety of chemical changes. Among these chemical reactions is one that is of particular value because it gives rise to colored compounds; namely, the ability of diazonium compounds to combine (couple) with imidazoles, phenols, and amines in alkaline solutions. *p*-Phenyldiazonium sulfonate, usually called diazobenzenesulfonic acid, is particularly valuable for colorimetric determinations because it is comparatively stable in water solutions, it does not couple with itself to give a highly colored compound, it is easy to prepare, the dyes formed from it are sufficiently soluble in alkaline solutions to render the use of a colorimeter possible, and the colors produced, usually orange to red, are easily compared because they do not readily produce retinal fatigue. It is not surprising then, to find that *p*-phenyldiazonium sulfonate has been used for some time as a qualitative test for imidazoles, phenols, and amines.[2, 3] Up to the present time two methods have been proposed for the use of this diazonium salt in the quantitative estimation of imidazoles;[4, 5]

[1] Griess, P., *Ann. chem. Pharm.*, 1866, cxxxvii, 39.

[2] Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

[3] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

[4] Weisz, M., and Ssobolew, N., *Biochem. Z.*, 1913–14, lviii, 119.

[5] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

but as far as is known to the authors this reagent has not been used, heretofore, for the quantitative estimation of phenols. This paper contains a detailed description of methods for the microchemical colorimetric determination of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine.

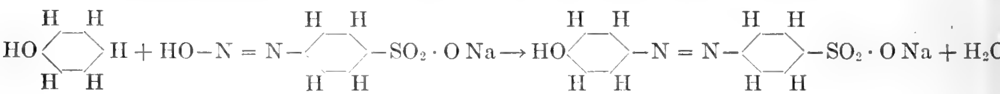
From the standpoint of color development, these phenols can be divided into three classes; namely,

1. Phenols in which the para position is not occupied by a second substituent.

2. Phenols in which the para position is occupied by a second substituent *that does not contain an amino group.*

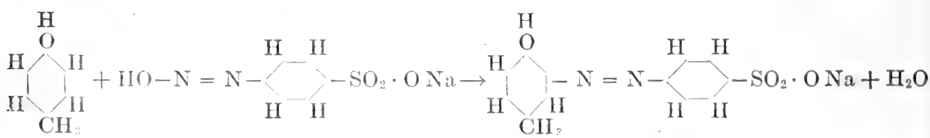
3. Tyrosine and tyramine.

When the position *para* to the phenol radical *is not occupied,* as in phenol, *o*-cresol, and *m*-cresol, the substitution with *p*-phenyldiazonium sulfonate occurs, at least predominantly, in this position.



The coupling is so rapid that about 95 per cent of the color has appeared before a reading can be taken with a Duboscq colorimeter. The color is fully developed in about 2 minutes and it does not change for about 3 to 5 minutes. In these cases the color is predominantly *yellow* with just a dash of red.

When the position para to the phenol radical *is occupied,* as in *p*-cresol and the aromatic hydroxy-acids, the substitution with *p*-phenyldiazonium sulfonate must occur in the ortho position.



The initial rate of coupling in this case, is somewhat slower than that of phenol; but a color of maximum intensity is,nevertheless, obtained in about 3 minutes. The colors produced are predominantly red so that a neutral solution of Congo red can serve as a comparison standard. These colors are stable for from 3 to 5

minutes. The stability of the color and the character of the change that the color undergoes after it has reached its maximum intensity is a fairly accurate index of the composition of the aromatic hydroxy-acid that is present, if only one of these acids is present. This is discussed in detail in the experimental section of the paper.

Tyrosine and tyramine, in small concentrations, show an anomalous behavior toward *p*-phenyldiazonium sulfonate in alkaline solutions. When a solution of tyrosine or tyramine is first added to the alkaline reagent, a pink color begins to develop promptly as in the case of the aromatic hydroxy-acids. After about 30 seconds, however, the color changes sharply to yellow and fades. The intensities of the yellow colors so produced are not directly proportional to the amount of tyrosine or tyramine present. Obviously, then, the usual procedure is worthless for the estimation of either tyrosine or tyramine.

The sharp change in color from pink to yellow suggested a chemical change, perhaps a tautomeric shift. In an effort to stabilize the pink color, or rather to revive it after the initial reaction had gone to completion, we added a small amount of a concentrated solution of sodium hydroxide. The color was intensified and some of the pink tint was restored; but again the color intensities were not directly proportional to the concentration of the phenol. The phenomena noted were so similar to those that one would expect from a compound in which a tautomeric equilibrium existed between a carbonyl form and an enol form that we thought to stabilize the carbonyl derivative, which we believed to be most highly colored, by allowing it to react with hydroxylamine to form an oxime. The addition of hydroxylamine hydrochloride to the alkaline reagent did *not* give rise to an intensification of color; but when sodium hydroxide was added previous to the addition of hydroxylamine, *a very intense bluish red color was produced which was directly proportional to the amount of tyrosine or tyramine added.* Briefly then, the method for estimating either tyrosine or tyramine consists of

1. A reaction between tyrosine or tyramine and *p*-phenyldiazonium sulfonate in an alkaline—sodium carbonate—solution which gives rise to a primary yellow color. This reaction is allowed to proceed for 5 minutes.

2. The addition of 2 cc. of 3.0 N NaOH, after the initial reaction period of 5 minutes, which intensifies the color, stops any further action between the phenol and the diazonium salt and converts the diazonium salt into a sodium diazotate which cannot couple with the hydroxylamine that is to be added later. The alkali is allowed to act for 1 minute.

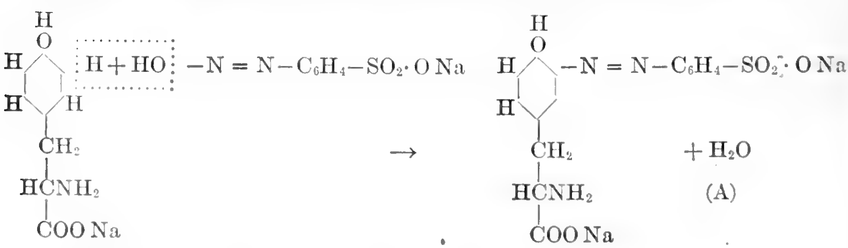
3. The addition of 0.10 cc. of a 20 per cent solution of hydroxylamine hydrochloride, which gives rise to a very intense bluish red color that is stable for at least half an hour.

An explanation of the chemical reactions involved must account for the following facts: Tyrosine and tyramine are the only phenols, that we have examined, that give a color intensification with sodium hydroxide and hydroxylamine. Since oxyphenyllactic acid, which is structurally identical with tyrosine excepting that an OH group replaces the NH_2 group, does not give a color intensification with NaOH and NH_2OH, *the NH_2 group must play a rôle in the color development with these phenols.*

A number of other compounds in which a tautomeric equilibrium exists between a carbonyl form and an enol form, for example acetaldehyde, acetone, and acetoacetic acid, also give an intense color under the conditions just described. The color produced is almost identical with that obtained with tyrosine or tyramine.

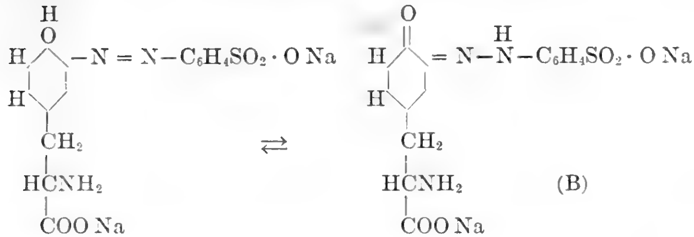
We offer the following explanation tentatively for these color phenomena. Work is now under way to determine the correctness of the following formulations.

Since the position para to the phenol group in tyrosine and tyramine is occupied by an alanyl side chain, the substitution with diazobenzenesulfonic acid must occur in the ortho position.



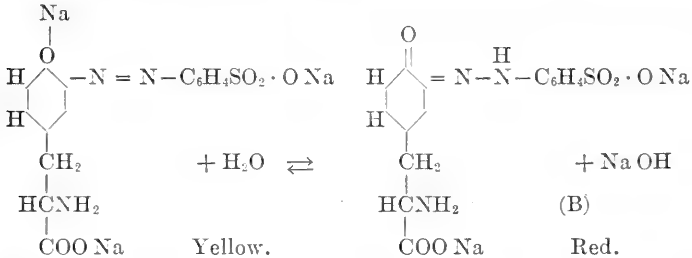
(A)

Compound A—the yellow phenol form—would be in tautomeric equilibrium with a small amount of the supposedly red quinoid form



(B)

In a solution made alkaline with sodium carbonate, practically all of the compound would exist in its phenol form A. The addition of a strong alkali, like sodium hydroxide, would give rise to the sodium phenate derivative of A which, because of its high degree of dissociation, would pass more easily into the quinoid derivative than the phenol itself.



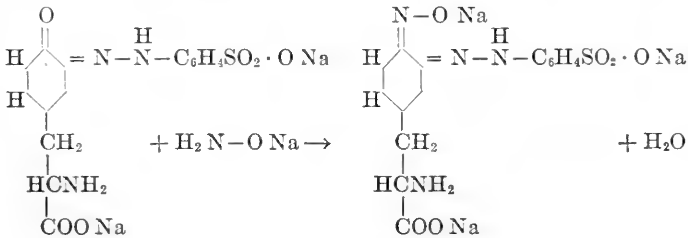
(B)

Yellow. Red.

The result would be an intensification of color with the influx of red. The amount of quinoid derivative formed under the influence of alkali must, nevertheless, be *small* because the color intensification is not very great. The addition of hydroxylamine hydrochloride to such a strongly alkaline solution would give rise to the following reactions.

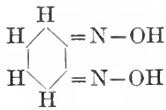
1. $NH_2OH{\cdot}HCl + 2\,NaOH \rightarrow H_2N{-}O\,Na + NaCl + H_2O$

2.



One would expect the quinoneoximehydrazone derivative (C) formed in this way to be dark red. Such *o*-quinoneoximehydrazones seem not to have been studied; but the para derivatives have been known for some time.[6] *They all give deep red alkaline solutions.*

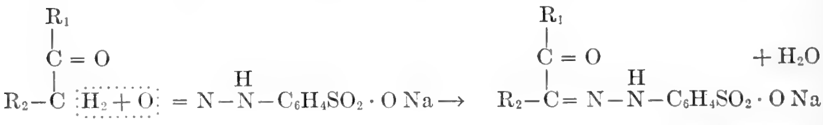
o-Quinonedioxime



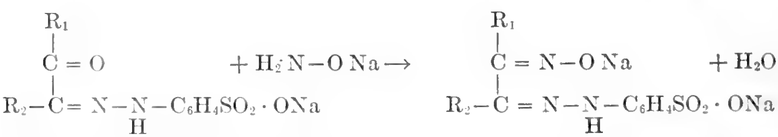
which is a closely related compound, also gives a deep red alkaline solution.[7]

Just why tyrosine should give a highly colored quinoneoximehydrazone while *p*-oxyphenyllactic acid does not give such a highly colored derivative, is a problem for future investigation. The rôle played by the amino group in the side chain of tyrosine and tyramine is still obscure.

The reactions that probably occur when certain aldehydes and ketones are treated with diazobenzenesulfonic acid, sodium hydroxide, and hydroxylamine can be represented by the following type equations.



Pale yellow.



We believe that the red compounds formed with acetaldehyde, acetone, and acetoacetic acid are the sodium salts of hydrazoxime derivatives. Von Pechmann and Wehsarg[8] have prepared a hydrazoxime derivative of methyl glyoxal which is similar to the compound that we believe to be formed with acetone.

[6] Borsche, W., *Ann. chem. Pharm.*, 1907, cccIvii, 171.
[7] Hantzsch, A., and Glover, W. H., *Ber. chem. Ges.*, 1907, xl, 4344.
[8] von Pechmann, H., and Wehsarg, K., *Ber. chem. Ges.*, 1888, xxi, 2996.

$$\begin{array}{l} \text{H} \\ \text{C} = \text{N}-\text{OH} \\ | \\ \text{C} = \text{N}-\text{NH}-\text{C}_6\text{H}_5 \\ | \\ \text{CH}_3 \end{array} \qquad\qquad \begin{array}{l} \text{H} \quad\;\;\;\; \text{H} \\ \text{C} = \text{N}-\text{N}-\text{C}_6\text{H}_4-\text{SO}_2\cdot\text{OH} \\ | \\ \text{C} = \text{N}-\text{OH} \\ | \\ \text{CH}_3 \end{array}$$

von Pechmann and Wehsarg's hydrazoxime. — Hydrazoxime formed from acetone.

They give no data on the color of the compound in alkaline solutions although they found the alcoholic solution to be yellow and the sulfuric acid solution to be dark red.

EXPERIMENTAL.

Reagents Employed.

Most of the reagents employed in these estimations are identical with those employed by us in the estimation of imidazoles.[5] A detailed description is repeated here for the sake of completeness.

Stock Sulfanilic Acid.—Sulfanilic acid (4.5 gm.) is mixed with 45 cc. of 37 per cent hydrochloric acid (sp. gr. 1.19) in a 500 cc. volumetric flask. Water is then added to the mark. The solid dissolves slowly; but completely.

Stock Sodium Nitrite.—25 gm. of 90 per cent sodium nitrite are dissolved in water and diluted to 500 cc. in a volumetric flask.

Sodium Carbonate.—Baker and Adamson's anhydrous sodium carbonate (5.50 gm.) is dissolved in water and diluted to exactly 500 cc. We recommend the above grade because we have found it to give uniform results. The purity of this carbonate is a very important factor in the color development. Some grades of carbonate contain impurities that give yellow colors of inferior intensities. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali. Pyrex glass vessels have proved to be entirely satisfactory.

Stock Methyl Orange.—Vacuum-dried Grübler's methyl orange (0.5000 gm.) is dissolved in water and diluted to exactly 500 cc. This solution keeps indefinitely.

Stock Congo Red.—Vacuum-dried Grübler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volumet-

ric flask. Water is then added to the mark. This solution keeps indefinitely.

Stock Acid Fuchsin.—Vacuum-dried Harmer's acid fuchsin (2.5000 gm.) is dissolved in water and diluted to exactly 500 cc. in a volumetric flask. The thymol-preserved solution keeps indefinitely.

Stock Phenol Red.—Hynson, Westcott and Dunning's phenol red (0.0500 gm.) is dissolved in water and diluted to exactly 500 cc. The thymol-preserved aqueous solution can be kept for at least 1 year. The alcoholic solution employed by many investigators for pH determinations deteriorates quite rapidly and cannot be used in this work.

Standard Indicator Solutions.—For the estimation of phenol, a solution containing 10 cc. of stock phenol red in a total aqueous volume of 100 cc. is employed. Redistilled water should be used for dilutions and the volumetric flask should be thoroughly rinsed with distilled water before the phenol red solution is introduced. It is best to prepare a fresh standard every day. The color so obtained matches that produced by phenol perfectly. When this standard indicator solution has been used for comparisons the symbol (Ph−R) is suffixed to the reading obtained.

For the estimation of o- and m-cresol, a solution containing 5 cc. of stock methyl orange in a total aqueous volume of 500 cc. is employed. The color so obtained matches that produced by *o*- and *m*-cresol almost perfectly. When this standard indicator solution has been used for comparisons the symbol (MO) is suffixed to the reading obtained.

For the estimation of p-cresol, and p-oxyphenylacetic, p-oxyphenylpropionic, and p-oxyphenyllactic acids, a solution containing 1.00 cc. of stock Congo red in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (CR) is suffixed to the reading obtained.

For the estimation of tyrosine or tyramine, a solution containing 1 cc. of stock acid fuchsin and 1.8 cc. of stock methyl orange in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (F−MO) is suffixed to the reading obtained.

Preparation of p-Diazobenzenesulfonic Acid Solution (The Reagent).

1.50 cc. each of the stock sulfanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6.00 cc. more of the stock sodium nitrite solution are added and the well mixed solution is again allowed to lie in the ice bath for 5 minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least 15 minutes after diluting with water. We have found it to give perfect results after 24 hours. It is best, however, to prepare a fresh reagent every day.

Preparation of the Phenols.

Phenol.—Merck's highest purity phenol was distilled *in vacuo.* The product collected boiled at 95° in a vacuum of 10 mm. of Hg. The colorless oil solidified readily. The colorless crystals so obtained had a melting point of 43°. The substance was assumed to be 100 per cent pure phenol.

o-Cresol.—A chemically pure product obtained from a local supply house was doubly distilled *in vacuo.* The colorless oil obtained boiled at 81° at a pressure of 10 mm. and at 188° under atmospheric pressure. The oil solidified on standing. The colorless crystals so obtained had a melting point of 32°. It was assumed to be 100 per cent pure *o*-cresol.

m-Cresol.—A chemically pure product obtained from a local supply house was doubly distilled *in vacuo.* The colorless oil so obtained boiled at 104° under 10 mm. pressure and at 201° under atmospheric pressure. It did not solidify. It was assumed to be 100 per cent pure *m*-cresol.

p-Cresol.—The *p*-cresol was obtained as a by-product in the preparation of tyramine.[9] The substance boiled at 90–91° at a pressure of 10 mm. and at 198°under atmospheric pressure. The oil solidified after standing for some time. The colorless crystals so obtained melted at 36°. It was assumed to be 100 per cent pure *p*-cresol.

[9] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 585.

Oxyphenylacetic Acid.—This substance was obtained as a by-product in the preparation of tyramine.[9] The colorless solid had the following properties.

1. It melted at 150°.
2. It left no residue on ignition.
3. The solid (0.1000 gm.) was dissolved in 10 cc. of water and titrated with 0.1 N NaOH using phenolphthalein as indicator. Exactly 6.59 cc. of the alkali were required to produce the first change in the indicator. The amount demanded by the formula is 6.58 cc. The substance was, therefore, 100 per cent pure.

Oxyphenylpropionic Acid.—This compound was prepared synthetically as follows.

Cinnamic acid was reduced, with sodium amalgam, to hydrocinnamic acid.[10]

p-Nitrophenylpropionic acid was then prepared by a modification of the method of Beilstein and Kuhlberg.[11]

Stöhr[12] prepared *p*-aminophenylpropionic acid by reducing the ethyl ester of *p*-nitrophenylpropionic acid with zinc and hydrochloric acid. He isolated the zinc double salt of the acid and treated this with acid and sodium nitrite to prepare *p*-oxyphenylpropionic acid. We reduced *p*-nitrophenylpropionic acid (6 gm.) directly with zinc (14 gm.) and hydrochloric acid (50 cc. of the 37 per cent acid) in alcoholic solution (70 cc. of 95 per cent alcohol) adding the acid slowly and keeping the temperature below 30°. The colorless liquid obtained, after the reaction had been allowed to proceed for 24 hours, was treated with 23.5 cc. of 95 per cent sulfuric acid and subjected to a distillation *in vacuo* at 60°. The pale yellow solid so obtained was dissolved in water (200 cc.) treated with sulfuric acid (20 cc. of the 95 per cent acid) and cooled in an ice bath. Sodium nitrite (60 cc. of a 5 per cent solution) was poured into the cold liquid. The resulting solution was allowed to remain in the ice bath for 1 hour after which it was treated with 250 cc. of water and heated to the boiling point. There was a copious evolution of nitrogen; but practically no tar formation. The aqueous solution was

[10] Fischer, E., Anleitung zur Darstellung organischer Präparate, Brunswick, 8th edition, 1908, 39.

[11] Beilstein, F., and Kuhlberg, A., *Ann. chem. Pharm.*, 1872, clxiii, 132.

[12] Stöhr, C., *Ann. chem. Pharm.*, 1884, ccxxv, 60.

cooled and extracted with ether. Removal of the ether, by distillation, left a pale yellow crystal cake weighing 4.5 gm. which was recrystallized first from water and then from hot benzene. The colorless solid finally obtained had the following properties.

1. It melted sharply at 130° (corrected).
2. It left no residue on ignition.
3. 0.166 gm. of the vacuum-dried solid was dissolved in water and the solution titrated with 0.20 N NaOH using phenolphthalein as indicator. Exactly 5.00 cc. of alkali were required. This is the amount demanded by the formula. The solid was 100 per cent pure.

p-Oxyphenyllactic Acid.—This was prepared from tyrosine by the method of Kotake.[13] The colorless solid obtained after drying to constant weight at 105° had the following properties.

1. It melted sharply at 172° (corrected).
2. It left no residue on ignition.
3. 0.25 gm. was dissolved in water and the solution titrated with 0.1 N NaOH using phenolphthalein as indicator. Exactly 13.70 cc. of alkali were required, which is almost exactly the amount (13.74 cc.) required by the formula. The solid was 100 per cent pure.

Tyrosine.—The tyrosine used in this work was prepared from horn. The pure white, asbestos-like solid had the following properties.

1. It did not melt at a temperature of 290°C.
2. It was free from ammonia.
3. It left no residue on ignition.
4. It contained no cystine.
5. The ammonia obtained from 0.1000 gm. of the solid—Kjeldahl method—neutralized 5.50 cc. of 0.10 N HCl as compared with 5.52 cc. required by the formula. The substance was 100 per cent pure.

Tyramine Hydrochloride.—The synthetic preparation and properties of this compound have been previously described by us.[9] The product used was 98.14 per cent pure tyramine hydrochloride. The impurity consisted entirely of sodium chloride.

[13] Kotake, Y., *Z. physiol. Chem.*, 1910, lxv, 398.

Procedure for Estimating Phenols Other than Tyrosine or Tyramine.

Process I.

The method used in developing the tables as well as the general procedure for estimating phenols other than tyrosine and tyramine is illustrated by the following example.

(1–X) cc. of water and 5 cc. of the 1.1 per cent sodium carbonate solution are accurately measured into the right-hand cylinder of a Duboscq colorimeter. 2 cc. of reagent are measured into a 5 second delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. X cc. of the phenol solution is allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed *thoroughly* as above.[14] The test cylinder is then transferred to the colorimeter and set at 20 mm. The left-hand cylinder, which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained.

The most accurate readings can be obtained by choosing such an amount of phenol solution that the standard indicator-containing cylinder has to be set at from 5 to 20 mm.

The method described here can be used on quantities of the phenol-containing solution varying from 0.01 to 1 cc. The combined volume of water and phenol solution used should always be 1 cc. Thus, if 0.10 cc. of the phenol solution is to be used, 0.90 cc. of water is added to the test cylinder. Then X equals 0.10 cc. and 1–X equals 0.90 cc.

Estimation of Small Amounts of Phenol.

A stock 1.00 per cent phenol solution was prepared by dissolving 2.0000 gm. of the pure solid in water and diluting to exactly 200 cc. From this the standard solution was prepared by dilut-

[14] Process II described on page 257 is the same as Process I up to this point.

ing 1 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of phenol.

The color produced with phenol is very intense. It develops so rapidly that it has reached 95 per cent of its maximum value before a reading can be taken. A maximum color is obtained within 2 minutes of the time that the phenol is added to the alkaline reagent. The color remains of maximum intensity for about 5 minutes, then fades slowly without changing appreciably in tint. Most people find this greenish yellow color hard to compare, without practice, because of the speed with which it produces retinal fatigue. Accurate readings can only be obtained by observing the colors briefly, setting the cylinder rapidly and making the fine adjustment after a brief interval of rest. When this is done the readings can be checked with an accuracy of about 1 per cent.

The previously described (Ph–R) standard indicator solution was used for comparisons in the compilation of Table I. This table shows clearly that the color production is directly proportional to the amount of phenol used.

Where only a few determinations are to be carried out it is usually simpler to compare the color produced by an unknown with that produced by a measured amount of a standard phenol solution, the two colors being prepared simultaneously. When the amount of standard phenol used is nearly equal to that in the test liquid, very accurate results can be obtained by this method.

Estimation of Small Amounts of o-Cresol.

A stock 0.1 per cent *o*-cresol solution was prepared by dissolving 1.0000 gm. of the pure solid in water and diluting to exactly 1,000 cc. From this the standard solution was prepared by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *o*-cresol.

The color produced with *o*-cresol is very intense. Its speed of development is like that of phenol. The color, which is predominantly yellow but which contains slightly more pink than that produced by phenol, remains of maximum intensity and permanent tint for about 3 minutes; then it fades rapidly and acquires a redder tint. This color is easier to compare than that of phenol.

TABLE I.

Estimation of Small Amounts of Phenol.

Depth of indicator solution (Ph—R) required to match the color in the test cylinder.	Phenol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.3	0.000002
3.5	0.000003
4.6	0.000004
5.8	0.000005
6.9	0.000006
8.1	0.000007
9.2	0.000008
10.4	0.000009
11.5	0.000010
12.7	0.000011
13.8	0.000012
15.0	0.000013
16.1	0.000014
17.3	0.000015
18.4	0.000016
19.6	0.000017
20.7	0.000018
21.9	0.000019
23.0	0.000020
24.2	0.000021
25.3	0.000022
26.5	0.000023
27.6	0.000024
28.8	0.000025
29.9	0.000026
31.1	0.000027
32.2	0.000028
33.4	0.000029
34.5	0.000030

The previously described (MO) standard indicator solution was used for comparisons in the compilation of Table II. This table shows clearly that the color produced is directly proportional to the amount of *o*-cresol used.

TABLE II.

Estimation of Small Amounts of o-Cresol.

Depth of indicator solution (MO) required to match the color in the test cylinder.	*o*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.4	0.000002
3.6	0.000003
4.8	0.000004
6.0	0.000005
7.3	0.000006
8.5	0.000007
9.7	0.000008
10.9	0.000009
12.1	0.000010
13.3	0.000011
14.5	0.000012
15.7	0.000013
16.9	0.000014
18.1	0.000015
19.4	0.000016
20.6	0.000017
21.8	0.000018
23.0	0.000019
24.2	0.000020
25.4	0.000021
26.6	0.000022
27.8	0.000023
29.0	0.000024
30.2	0.000025
31.5	0.000026
32.7	0.000027
33.9	0.000028
35.1	0.000029
36.3	0.000030

Estimation of Small Amounts of m-Cresol.

A stock 0.10 per cent solution of *m*-cresol was prepared by dissolving 1.000 gm. of the colorless oil in water and diluting to exactly 1,000 cc. From this the standard solution was prepared

by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *m*-cresol.

TABLE III.

Estimation of Small Amounts of m-Cresol.

Depth of indicator solution (MO) required to match the color in the test cylinder.	*m*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.0	0.000001
1.9	0.000002
2.9	0.000003
3.9	0.000004
4.9	0.000005
5.8	0.000006
6.8	0.000007
7.8	0.000008
8.7	0.000009
9.7	0.000010
10.7	0.000011
11.6	0.000012
12.6	0.000013
13.6	0.000014
14.6	0.000015
15.5	0.000016
16.5	0.000017
17.5	0.000018
18.4	0.000019
19.4	0.000020
20.4	0.000021
21.3	0.000022
22.3	0.000023
23.3	0.000024
24.3	0.000025
25.2	0.000026
26.2	0.000027
27.2	0.000028
28.1	0.000029
29.1	0.000030

The intense color produced with *m*-cresol is qualitatively identical with that obtained with *o*-cresol. In this case, however,

the time of complete development is about 5 minutes although most of the color appears immediately. At first the color is slightly more yellow than the (MO) comparison standard. In the course of 3 minutes the match is perfect and a maximum of color is obtained in 5 minutes. The color fades slowly and becomes pinker. The previously described (MO) standard indicator solution was used for comparisons in the compilation of Table III. This table shows clearly that the color production is directly proportional to the amount of *m*-cresol used.

Estimation of Small Amounts of p-Cresol.

A stock 0.10 per cent solution of *p*-cresol was prepared by dissolving 1.000 gm. of the colorless solid in water and diluting to exactly 1,000 cc. From this the standard solution was prepared by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-cresol.

In this case the color, which is predominantly red, develops to its maximum intensity during the 30 seconds required to mix the contents of the test cylinder. The readings recorded are those obtained *immediately* after the test cylinder was transferred to the colorimeter. The comparison must be made within 2 minutes during which time the color remains of constant and maximum intensity. Then the test liquid, although it changes but little in intensity, rapidly acquires a cloudy appearance which renders further comparisons untrustworthy. This same cloudy appearance is also obtained when the test liquid contains a finely divided precipitate which leads us to believe that an insoluble compound appears, after 2 minutes, although none can be seen with the naked eye.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table IV. As can be seen from the table, the color production is directly proportional to the amount of *p*-cresol used.

Estimation of Small Amounts of p-Oxyphenylacetic Acid.

A stock 1.00 per cent solution of *p*-oxyphenylacetic acid was prepared by dissolving 1.0000 gm. of the colorless solid in water and diluting to 100 cc. This solution was preserved with chloroform.

From it, the standard solution was prepared by diluting 1 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenylacetic acid.

TABLE IV.

Estimation of Small Amounts of p-Cresol.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
2.5	0.000005
3.0	0.000006
3.5	0.000007
4.0	0.000008
4.5	0.000009
5.0	0.000010
5.5	0.000011
6.0	0.000012
6.5	0.000013
7.0	0.000014
7.5	0.000015
8.0	0.000016
8.5	0.000017
9.0	0.000018
9.5	0.000019
10.0	0.000020
10.5	0.000021
11.0	0.000022
11.5	0.000023
12.0	0.000024
12.5	0.000025
13.0	0.000026
13.5	0.000027
14.0	0.000028
14.5	0.000029
15.0	0.000030

In this case the color, which is predominantly red, develops to its maximum intensity in about 2 minutes. This color of maximum intensity is permanent for about 5 minutes after which it fades slowly and becomes yellow.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table V. It has a color that is slightly more red than that produced by *p*-oxyphenylacetic acid; but the match is so close that accurate comparisons are easily made. As can be seen from Table V the color production is directly proportional to the amount of *p*-oxyphenylacetic acid used.

TABLE V.

Estimation of Small Amounts of p-Oxyphenylacetic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenylacetic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
3.6	0.000010
4.0	0.000011
4.3	0.000012
4.7	0.000013
5.0	0.000014
5.4	0.000015
5.7	0.000016
6.1	0.000017
6.5	0.000018
6.8	0.000019
7.2	0.000020
7.6	0.000021
7.9	0.000022
8.3	0.000023
8.6	0.000024
9.0	0.000025
9.4	0.000026
9.7	0.000027
10.1	0.000028
10.4	0.000029
10.8	0.000030

Estimation of Small Amounts of p-Oxyphenylpropionic Acid.

A stock 1.00 per cent solution of *p*-oxyphenylpropionic acid was prepared by dissolving 1.0000 gm. of the solid in 30 cc. of 0.20 N NaOH and diluting, with water, to exactly 100 cc. Chloroform was added as a preservative. The standard solution was prepared from the stock solution by diluting 1.00 cc. of the latter

to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenylpropionic acid. The color, which is predomi-

TABLE VI.

Estimation of Small Amounts of p-Oxyphenylpropionic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenylpropionic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
3.1	0.000010
3.4	0.000011
3.7	0.000012
4.0	0.000013
4.3	0.000014
4.6	0.000015
5.0	0.000016
5.3	0.000017
5.6	0.000018
5.9	0.000019
6.2	0.000020
6.5	0.000021
6.8	0.000022
7.1	0.000023
7.4	0.000024
7.7	0.000025
8.1	0.000026
8.4	0.000027
8.7	0.000028
9.0	0.000029
9.3	0.000030
9.6	0.000031
9.9	0.000032
10.2	0.000033
10.5	0.000034
10.8	0.000035
11.1	0.000036
11.5	0.000037
11.8	0.000038
12.1	0.000039
12.4	0.000040

nantly red, develops to its maximum intensity in about 2 minutes and undergoes no change for from 1 to 3 minutes depending

upon the quantity of p-oxyphenylpropionic acid present and the room temperature. This period of color constancy is followed, rather sharply, by a rapid decline in color intensity, a cloudy appearance and a change in tint. The values recorded in Table VI are those obtained just previous to this change.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table VI. This table shows that the color production is directly proportional to the amount of p-oxyphenylpropionic acid present.

Estimation of Small Amounts of p-Oxyphenyllactic Acid.

A stock 1.00 per cent solution of p-oxyphenyllactic acid was prepared by dissolving 1.0000 gm. of the pure solid in 56 cc. of 0.1 N NaOH and diluting with water to exactly 100 cc. Chloroform was added as a preservative. The standard solution was prepared by diluting 1.00 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of p-oxyphenyllactic acid.

The color, which is predominantly red, develops to its maximum intensity in about 4 minutes and undergoes no change for about 5 minutes. The (CR) standard, which was used for comparisons in the compilation of Table VII, is slightly more red than the color produced by p-oxyphenyllactic acid; but the discrepancy in tint is too slight to interfere with the accuracy of the comparisons. The slowness of the color development, the slight yellow tint, and the stability of the color are characteristics for oxyphenyllactic acid. In this case there is no sharp color change and the liquid does not become cloudy as in the case of oxyphenylpropionic acid.

The tabular values have been carried out to the second place because these figures are obviously more correct than the actual readings which can, of course, be obtained with an accuracy of only one decimal place. Thus for 0.000019 gm. of p-oxyphenyllactic acid a reading of either 4.70 or 4.80 mm. is obtained; but the correct reading would be 4.75. This figure (4.75) therefore, appears in the table. As in the case of the other phenols, the color production is directly proportional to the amount of the phenol used.

TABLE VII.

Estimation of Small Amounts of p-Oxyphenyllactic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenyllactic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
2.5	0.000010
2.75	0.000011
3.0	0.000012
3.25	0.000013
3.5	0.000014
3.75	0.000015
4.0	0.000016
4.25	0.000017
4.5	0.000018
4.75	0.000019
5.0	0.000020
5.25	0.000021
5.5	0.000022
5.75	0.000023
6.0	0.000024
6.25	0.000025
6.5	0.000026
6.75	0.000027
7.0	0.000028
7.25	0.000029
7.5	0.000030
7.75	0.000031
8.0	0.000032
8.25	0.000033
8.5	0.000034
8.75	0.000035
9.0	0.000036
9.25	0.000037
9.5	0.000038
9.75	0.000039
10.0	0.000040
10.25	0.000041
10.5	0.000042
10.75	0.000043
11.0	0.000044
11.25	0.000045
11.5	0.000046
11.75	0.000047
12.0	0.000048
12.25	0.000049
12.5	0.000050

Procedure for the Estimation of Small Amounts of Tyrosine and Tyramine.

Process II.

As has already been pointed out in the introduction, tyrosine and tyramine show an anomalous behavior toward *p*-phenyldiazonium sulfonate in alkaline—sodium carbonate—solutions. When a solution of tyrosine or tyramine is added to the alkaline reagent, a pink color develops promptly, as in the case of the aromatic hydroxy-acids. After about 30 seconds, however, the color changes sharply to yellow and fades. The pink color is too evanescent to compare and the intensities of the yellow colors are not directly proportional to the amount of tyrosine or tyramine used. The procedure that has been found to give satisfactory results with the other phenols, and with imidazoles, cannot be used for the estimation of tyrosine or tyramine. The procedure finally adopted is given below; the reasons for its adoption and an explanation of the chemical reactions involved have already been given in the introduction.

Process I described for the other phenols (see page 246) is followed as far as foot-note 14. The test cylinder is allowed to stand for exactly $5\frac{1}{2}$ minutes from the time that the tyrosine or tyramine solution began to mix with the alkaline reagent. This gives rise to a primary color that is yellow to orange and whose intensity is not directly proportional to the amount of the phenol used.

Sodium hydroxide (2.00 cc. of a 3.0 N solution) is added and the contents of the cylinder thoroughly mixed as before. This gives rise to a marked color intensification with a change of tint toward red, the color still being, however, predominantly yellow.

Exactly 1 minute after the sodium hydroxide solution began to mix with the liquid in the test cylinder, 0.10 cc. of a 20 per cent solution of hydroxylamine hydrochloride[15] is rapidly introduced and the contents of the cylinder are again thoroughly and rapidly mixed. At first there is no change in color. Then suddenly, after a latent period of from 5 to 10 seconds, an intense bluish red color

[15] This was obtained from the Special Chemicals Co., Highland Park, Illinois.

develops. This secondary color develops to its full intensity while the cylinder and contents are being agitated (30 seconds) and changes very little in tint or intensity in half an hour. It is best to introduce the hydroxylamine solution from a rapid delivery 0.10 cc. pipette and to have the liquid well mixed before the secondary color *begins* to develop. The test cylinder is transferred to the right-hand side of the Duboscq colorimeter and set at 25 mm. The left-hand cylinder, which contains the previously described (F–MO) comparison standard, is then adjusted until the two halves of the field are identical in tint and intensity.

The color is easily compared and the determinations are accurate to about 0.5 to 1.5 per cent. If the process is properly carried out, the correction blank (see later) is about 0.30 mm. (F–MO). This amount must be subtracted from the actual reading to obtain the values recorded in Table VIII. We have found that a high laboratory temperature raises this correction blank from 0.10 to 0.20 mm. *The hydroxylamine solution must not be introduced until the sodium hydroxide has been allowed to react with the alkaline reagent for at least 1 minute;* otherwise a colored compound is produced with the hydroxylamine.

The tabular values are accurate for laboratory temperatures ranging from 18 to 25°. At lower temperatures the colors are very slightly less intense and at higher temperatures they are slightly more intense. It is always advisable, before carrying out a determination on an unknown, to run a few determinations on a standard tyrosine solution to be certain that the tabular values are accurate for the existing laboratory conditions.

It is a curious coincidence that the tabular values are identical for both tyrosine and tyramine hydrochloride. The same table may, therefore, be used in the estimation of either.

A stock 1.00 per cent solution of tyrosine was prepared by dissolving 2.0000 gm. of the pure solid in 75 cc. of 1.0 N HCl and diluting with water to 200 cc. From this the standard solution was prepared by diluting 1 cc. to 100 cc. in a volumetric flask. Each cc. of the standard solution contained 0.0001 gm. of tyrosine.

A stock 1.00 per cent solution of tyramine hydrochloride was prepared by dissolving 2.0379 gm. of the 98.14 per cent solid in water and diluting to exactly 200 cc. Chloroform was added as a preservative. The standard solution was prepared by diluting

1.00 cc. of the stock solution to 100 cc. in a volumetric flask. Each cc. of the standard solution contained 0.0001 gm. of tyramine hydrochloride.

TABLE VIII.

Estimation of Small Amounts of Tyrosine and Tyramine Hydrochloride.

Depth of indicator solution (F—MO) required to match the color in the test cylinder.	Tyrosine or tyramine hydrochloride in the test cylinder (total volume 10.1 cc.) test cylinder set at 25 mm.
mm.	*gm.*
4.0	0.000005
4.8	0.000006
5.6	0.000007
6.4	0.000008
7.2	0.000009
8.0	0.000010
8.8	0.000011
9.6	0.000012
10.4	0.000013
11.2	0.000014
12.0	0.000015
12.8	0.000016
13.6	0.000017
14.4	0.000018
15.2	0.000019
16.0	0.000020
16.8	0.000021
17.6	0.000022
18.4	0.000023
19.2	0.000024
20.0	0.000025
20.8	0.000026
21.6	0.000027
22.4	0.000028
23.2	0.000029
24.0	0.000030

The Correction Blank.

When the reagent and alkali are mixed in the absence of a phenol derivative, a very pale yellow color is produced in the course of 5 minutes. This color has an intensity value equivalent to about 0.30 mm. of any of the standard indicator solutions used. This

same amount of color is also produced in the presence of phenol derivatives along with the color produced by the phenol; so the readings obtained are high by about 0.30 mm. in every case. It is necessary, therefore, to subtract 0.30 mm. from the readings obtained before comparison with the tables.

Substances That Do and Do Not Interfere with the Quantitative Determination of Phenols.

The tests for interference were carried out as follows, unless otherwise specified.

Tyrosine (1.00 cc. of the stock 1 per cent solution) was mixed, in a 100 cc. graduated precision cylinder, with quantities of the interfering substances as given below. Water was added to give a total volume of 100 cc. Then 0.10 and 0.20 cc. portions of this liquid were taken for the colorimetric determinations.

Interference with the determination of tyrosine was studied particularly because this determination involves a heretofore unstudied type of reaction. Phenols other than tyrosine and tyramine are determined by a process identical with that used for imidazoles and the interference with that process has already been studied and reported.

Sodium and potassium chloride, sulfate, phosphate, acetate, and citrate in 5 per cent concentrations do not interfere with the colorimetric determination of tyrosine or tyramine.

Ammonium salts interfere very seriously with the determination. When the concentration of ammonium chloride is 5 per cent, so much of a greenish yellow color is produced that the *presence* of tyrosine would not be suspected. Needless to say, tyrosine could not be determined under these conditions. When the concentration of ammonium chloride is 1 per cent, the interference is still considerable because 0.10 cc. of the solution had a color value equal to 9.0 mm. (F–MO) as compared to a normal value of 8.0 mm.; which is a positive error of 12.5 per cent. The color was quite yellow. When the concentration of ammonium chloride is reduced to 0.5 per cent results were obtained that are fairly satisfactory for now 0.10 cc. had a color value equal to 8.2 mm. (F–MO) which is 102.5 per cent of the correct reading. The color was exactly like that of the standard. *Ammonium salts must not be present in concentrations exceeding 0.5 per cent in liquids that are to be examined for tyrosine colorimetrically.*

Amino-acids also interfere seriously with the colorimetric estimation of tyrosine as can be seen from the following data.

Leucine.—A solution was prepared containing 5 cc. of a 1 per cent leucine solution, 0.20 cc. of a 1 per cent tyrosine solution, and sufficient water to give a total volume of 10 cc. Of this solution

0.05 cc. had a color value equivalent to 10.6 mm. (F − MO) and
0.10 " " " " " " " 18.7 " (F − MO).

The color contained far more yellow than the comparison standard. Both of these values are high, the 0.05 cc. reading being 30 per cent high and the 0.10 cc. reading being 15 per cent high. This excess of color is due to a yellow compound that is formed when leucine reacts with *p*-phenyldiazonium sulfonate. In this case the ratio of leucine to tyrosine was 25 to 1.

A second liquid was prepared containing 1.00 cc. of a 1 per cent leucine solution, 0.10 cc. of a 1 per cent tyrosine solution, and sufficient water to give a total volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.4 mm. (F − MO) and
0.20 " " " " " " " 16.8 " (F − MO).

The color obtained contained slightly more yellow than the comparison standard; but the match was very good. These values are 5 per cent higher than they should be. The ratio of leucine to tyrosine in this case was 10 to 1.

These experiments show that *leucine interferes seriously with the colorimetic estimation of tyrosine when the ratio of leucine to tyrosine exceeds 10 to 1.*

Glycine.—A solution was prepared containing 0.30 cc. of a 1 per cent glycine solution and 0.10 cc. of a 1 per cent tyrosine solution in a total aqueous volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F − MO) and
0.20 " " " " " " " 16.0 " (F − MO).

The color produced matched that of the standard perfectly. The values obtained are exactly what they would have been if glycine had not been present. When the ratio of glycine to tyrosine is 3 to 1, the colorimetric estimation of tyrosine is not interfered with.

A second solution was prepared containing 0.50 cc. of a 1 per cent glycine solution and 0.10 cc. of a 1 per cent tyrosine solution in a total aqueous volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.5 mm. (F – MO) and
0.20 " " " " " " 17.0 " (F – MO).

The color produced was distinctly yellow and not easy to compare with the (F–MO) comparison standard. The values are too high by 6 per cent. Even at this ratio (5 to 1), glycine interferes seriously with the colorimetric determination of tyrosine. With higher concentrations of glycine the interference becomes so pronounced that a determination of any kind is impossible. The color produced is predominantly yellow so that the color due to tyrosine is masked almost completely.

These experiments show that *glycine interferes seriously with the colorimetric estimation of tyrosine when the ratio of glycine to tyrosine exceeds 5 to 1.* From these experiments it is clear that the direct determination of tyrosine colorimetrically in the phosphotungstate filtrate fraction of a protein is impossible by means of this method *as it now stands.* Tyrosine, excepting for that part which can be separated by crystallization, is always associated with a high percentage of other amino-acids. To estimate tyrosine under these conditions we have either to separate tyrosine, or some derivative into which it can be quantitatively converted, from the bulk of the other amino-acids, or to remove the interfering NH_2 groups of these other amino-acids without destroying the colorimetric properties of tyrosine. Attempts are now being made in this laboratory to modify this method so that it will be applicable to the estimation of tyrosine in proteins.

Hydrogen Peroxide.—A solution was prepared containing 0.01 gm. of tyrosine and 0.20 cc. of a 3 per cent commercial hydrogen peroxide solution in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 10.0 mm. (F – MO)

as compared to a normal value of 16.0 mm. The color contained far more yellow than the comparison standard. Hydrogen peroxide is, therefore, a very potent interfering substance.

In its presence extremely low and entirely unreliable values are obtained. Fortunately, hydrogen peroxide can be readily removed from an aqueous solution by treating it with platinized asbestos. An example of such a treatment is given below under formaldehyde.

Formaldehyde.—A liquid was prepared containing 0.01 gm. of tyrosine and 5 cc. of commercial formaldehyde in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 9.2 mm. (F − MO).

The reading should have been 16.0 mm. The color matched that of the comparison standard very well.

A liquid was now prepared containing 0.01 gm. of tyrosine and 2 cc. of formaldehyde in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 14.4 mm. (F − MO).

In this case the reading was low by 10 per cent.

Finally a solution was prepared containing 0.01 gm. of tyrosine and 1.00 cc. of formaldehyde in a total aqueous volume of 100 cc. of which

0.20 cc. had a color value equivalent to 16.0 mm. (F − MO).

This is the normal reading. In this concentration (1.0 per cent) formaldehyde *does not* interfere with the colorimetric estimation of tyrosine.

It is of interest to note that *low* values are obtained when formaldehyde is present. Formaldehyde does *not* give a colored substance with *p*-phenyldiazonium sulfonate. In this respect this aldehyde is very different from acetaldehyde, acetone, and acetoacetic acid since the latter substances give intensely colored compounds with *p*-phenyldiazonium sulfonate in alkaline solution after treatment with sodium hydroxide and hydroxylamine. *Formaldehyde cannot form an olefine enol,* it cannot give rise to hydrazoxime derivatives; the other three carbonyls can. This may account for their different behaviors.

Since it is conceivable that tyrosine determinations might have to be carried out on liquids containing formaldehyde, we sought

for a method that would destroy formaldehyde without injuring tyrosine. Blank and Finkenbeiner[16] have described a method for the quantitative conversion of formaldehyde into sodium formate by means of hydrogen peroxide. We found that this method destroyed formaldehyde and did not destroy tyrosine; but the hydrogen peroxide left after the reaction had gone to completion made it impossible to determine the tyrosine colorimetrically. We then removed the hydrogen peroxide by means of platinized asbestos and found that the concentration of tyrosine had not changed. The experiment was conducted as follows.

A solution was prepared containing 0.01 gm. of tyrosine, 7 cc. of water, 3 cc. of commercial formaldehyde, and 17 cc. of 3 N NaOH. The substances were mixed in a 100 cc. graduated cylinder. After a reaction period of about 15 minutes, hydrogen peroxide (50 cc. of a 3 per cent commercial preparation) was slowly added to the contents of the cylinder *without cooling.* After the evolution of gas had ceased (15 minutes) 0.30 gm. of a 5 per cent platinized asbestos was added to the liquid. The evolution of oxygen was practically nil after 30 minutes. The liquid was neutralized with 5 N sulfuric acid (which took 3 cc.) and diluted to 100 cc. Of the clear colorless liquid obtained after filtration,

0.10 cc. had a color value equivalent to 8.0 mm. (F – MO) and
0.20 " " " " " " 15.9 " (F – MO)

which is equivalent to 0.01 gm. of tyrosine for the entire test liquid, 100 per cent of the starting material. Formaldehyde and hydrogen peroxide do not destroy tyrosine under the conditions of the above experiment. Both of these interfering substances can be completely removed without interfering with the color-producing properties of tyrosine.

Carbonyl-Enol Interference (Acetaldehyde, Acetone, and Acetoacetic Acid).

These three substances have been classified as carbonyl-enols because they are capable of existing in two tautomeric forms; namely,

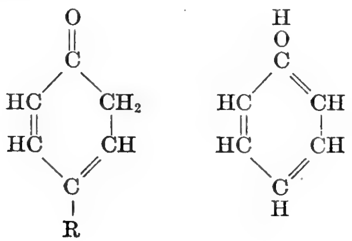
[16] Blank, O., and Finkenbeiner, H., *Ber. chem. Ges.*, 1898–99, xxxi, 2979.

Carbonyl form.	Enol form.
$CH_3-\underset{\displaystyle H}{\underset{\vert}{C}}{:}O$	$H_2C = \underset{\displaystyle H}{\underset{\vert}{C}}-OH$
$CH_3-\underset{\displaystyle O}{\underset{\Vert}{C}}-CH_3$	$CH_3-\underset{\displaystyle OH}{\underset{\vert}{C}} = CH_2$
$CH_3-CO-CH_2-COOH$	$CH_3-\underset{\displaystyle OH}{\underset{\vert}{C}} = \overset{\displaystyle H}{\overset{\vert}{C}}-COOH$

These compounds are particularly potent as interfering agents because they produce a color, under the conditions employed in the estimation of tyrosine, that closely resembles that produced by tyrosine. These colors are so intense that as little as 0.00005 gm. of any of the three substances gives sufficient color to be easily read in a colorimeter.

When the carbonyl derivative is first added to the alkaline reagent a brilliant red color is produced that fades in a few seconds and becomes yellow. The addition of sodium hydroxide, after the initial 5 minute reaction period, enhances this color and changes its tint to bluish red. The subsequent addition of hydroxylamine gives rise to an intense bluish red color that is almost identical with that obtained with tyrosine.

The fact that so simple a substance as acetaldehyde should give a color similar to that obtained with tyrosine would lead one to suspect that this carbonyl-enol tautomerism played a rôle in the color production by tyrosine and tyramine (see introduction for the chemical reactions involved). Phenols can also occur in two tautomeric forms that would properly be called carbonyl and enol, respectively



Acetone is the only member of this group upon which we have carried out any extended experiments. A stock 5 per cent solution of acetone was prepared by diluting 5 gm. of the pure product with water to 100 cc. A standard solution was then prepared by diluting 4 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.002 gm. of acetone. When 0.20 cc. of this standard solution (equivalent to 0.0004 gm. of acetone) was mixed with the alkaline reagent and subsequently treated with sodium hydroxide and hydroxylamine as in the tyrosine determination, a color was produced that was equivalent to about 8.5 mm. (F–MO), the color being slightly redder than that of the comparison standard. It was almost impossible to obtain exactly this value on repetition because the slightest change in the primary reaction time produced a large change in the reading finally obtained. We, therefore, tried several experiments (using 0.20 cc. of the standard acetone solution) in which this reaction time was elongated. With a reaction time of 15 minutes a color value of 29.6 mm. (F–MO) was obtained. With a reaction time of 25 minutes a color value of 31.6 mm. (F–MO) was obtained. Obviously then, the 5 minute reaction period that we found to be ample for the complete development of color in the case of tyrosine and tyramine is too short for acetone. We have made no attempt to change the conditions so that acetone would give a maximum constant color; but this could, no doubt, be done. We wish merely, to call attention to the fact that with 0.0004 gm. of acetone a color value of 29.6 mm. (F–MO) was obtained when the reaction period was 15 minutes; hence as little as 0.00004 gm. of acetone would give a distinctly perceptible color. This method might, therefore, be useful as a qualitative test for traces of acetone even though further experiments might prove that this method could not be made to give readings constant enough for quantitative work.

It seems hardly necessary to say that these carbonyl-enols can never really interfere with the estimation of tyrosine or tyramine because the carbonyl compounds can always be removed by distillation or evaporation.

Glucose.—Glucose and the other sugars containing a free aldehyde or ketone group would be expected to interfere with the colorimetric estimation of tyrosine because of the yellow color

that these compounds give with sodium hydroxide. When 0.10 cc. of a 5 per cent glucose solution is subjected to the treatment used for estimating tyrosine, a very pale yellow primary color is produced which changes to brown with marked intensification on the addition of sodium hydroxide and then becomes redder when hydroxylamine is added. The color finally obtained is still far too yellow to make a comparison with the (F–MO) standard possible. Tyrosine (0.01 gm.) dissolved in 100 cc. of a 5 per cent glucose solution, produces its own color just as it does in the absence of glucose; but the color finally obtained is the sum of the tyrosine and glucose colors. Thus, of this solution

0.10 cc. had a color value equivalent to 10.3 mm. (F – MO) and
0.20 " " " " " " 19.3 " (F – MO).

The colors obtained in each case contained more yellow than the comparison standard. If these readings are calculated as tyrosine, they are 20 and 13 per cent high, respectively.

Although so high a concentration of glucose would interfere seriously with the colorimetric estimation of tyrosine, lower concentrations are less potent. Thus a tyrosine solution containing 1 per cent of glucose gives a color whose tint and intensity are exactly like those of a solution containing no glucose. *In 1 per cent concentration glucose does not interfere with the estimation of tyrosine.*

Alcohols.—Methyl and ethyl alcohol in 10 per cent concentrations give rise to a color with *p*-phenyldiazonium sulfonate that resembles that produced by tyrosine. The colors are very faint, however, so that a positive interference of about 20 per cent is obtained with 10 per cent concentrations of alcohol. We believe that the pure alcohols do *not* interfere with the colorimetric process and that it is the acetone and acetaldehyde present in the alcohols that produce the color. Ethyl alcohol, that has been freshly distilled over potassium hydroxide augments the color of a tyrosine solution very little even when the alcohol concentration is 10 per cent.

An aqueous tyrosine solution, that has been saturated with amyl alcohol, gives values that are about 5 per cent too high. A chloroform extraction of this liquid *reduces* the reading to about

2 per cent above normal; but does not remove the amyl alcohol completely. Any of these alcohols can, of course, be completely removed by distillation or evaporation; so they can offer no permanent difficulty.

Chloroform, toluene, and ether that has been distilled over sodium, do not interfere with the colorimetric estimation of tyrosine.

Charcoal.—In our earlier work we found that animal or vegetable charcoal adsorbed appreciable quantities of imidazoles and we advised against the use of charcoal in any liquid that was to be tested quantitatively for imidazoles. The adsorption power of charcoal for phenols is far greater than for imidazoles as can be seen from the following data.

5 cc. each of the 1 per cent stock solutions of tyrosine, tyramine, and phenol were separately diluted to 100 cc. and treated with 1 gm. of Pfanstiehl's decolorizing charcoal. After 10 minutes of agitation the liquids were filtered and colorimetric estimations made on the clear filtrates.

Of the tyrosine solution,

0.10 cc. had a color value equivalent to 9.2 mm. (F − MO)

which is equivalent to 0.0115 gm. of tyrosine; hence 0.0385 gm. of tyrosine was adsorbed by 1 gm. of charcoal.

Of the tyramine solution,

0.10 cc. had a color value equivalent to 20.5 mm. (F − MO)

which is equivalent to 0.0256 gm. of tyramine hydrochloride; hence 0.0244 gm. of tyramine hydrochloride was adsorbed by 1 gm. of charcoal.

Of the phenol solution,

1.0 cc. had a color value equivalent to 18.4 mm. (Ph − R)

which is equivalent to 0.0016 gm. of phenol; hence 0.0484 gm. of phenol was adsorbed by 1 gm. of charcoal.

Barium sulfate precipitates do not adsorb phenols from a neutral or acid solution.

SUMMARY.

1. Methods have been devised for the *quantitative colorimetric estimation* of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine.

2. These methods are based upon the well known fact that phenols react with diazonium compounds in alkaline solutions to give colored derivatives. A freshly prepared solution of *p*-phenyldiazonium sulfonate is mixed with a dilute solution of sodium carbonate. A dilute solution of the phenol whose concentration is to be estimated is mixed with the alkaline reagent which gives rise to a primary color that is yellow to red depending upon the character of the phenol.

3. The phenols studied can be divided into three classes.

A. Phenols in which the para position is not occupied by a second substituent.

B. Phenols in which the para position is occupied by a second substituent that does not contain an amino group.

C. Tyrosine and tyramine.

Phenols belonging to Class A (phenol and *o*- and *m*-cresol), couple with great speed and give rise to yellow colors.

Phenols belonging to Class B (*p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids), couple more slowly than those belonging to Class A. The color produced is predominantly red.

Tyrosine and tyramine show an anomalous behavior toward alkaline (Na_2CO_3) *p*-phenyldiazonium sulfonate. An evanescent pink color is produced at first, which fades in 30 seconds to a yellow of inconstant intensity. The simple process employed for the estimation of imidazoles and the other phenols cannot, therefore, be used for the estimation of tyrosine and tyramine. The primary yellow color produced by tyrosine or tyramine is enhanced somewhat by the addition of sodium hydroxide. The colors produced are not directly proportional to the amount of phenol present. If this strongly alkaline liquid is now treated with a small amount of hydroxylamine hydrochloride, a very intense bluish red color is produced whose intensity is directly proportional to the amount of tyrosine or tyramine present (Process II).

4. Tables are given for the direct determination of quantities of these phenols ranging from 0.000001 to 0.00005 gm. The amount of the phenol derivative in any quantity of liquid can then be determined, by multiplication, with an accuracy of from 0.5 to 3 per cent.

5. The alkali salts of the common organic and inorganic acids do not interfere with either of the above two colorimetric processes.

Ammonium salts and amino-acids give an intense yellow color with the process used for the estimation of tyrosine and tyramine (Process II). High values are obtained if these nitrogen compounds are present in sufficient concentration.

Hydrogen peroxide and formaldehyde suppress the color production by tyrosine; hence in the presence of these compounds low values are obtained.

Acetaldehyde, acetone, and acetoacetic acid give rise to a color that is qualitatively identical with that obtained with tyrosine and tyramine. The colors are so intense that the possibility of using this method in the estimation of these carbonyl derivatives suggests itself.

The presence of the ordinary alcohols leads to high readings probably because of the presence of aldehydes or ketones in the alcohols.

1 gm. of vegetable charcoal adsorbs 0.0385 gm. of tyrosine, 0.0244 gm. of tyramine, and 0.0484 gm. of phenol from 100 cc. of an aqueous solution that originally contained 0.05 gm. of the above phenols.

STUDIES ON PROTEINOGENOUS AMINES.

XV. A QUANTITATIVE METHOD FOR THE SEPARATION AND ESTIMATION OF PHENOLS INCLUDING PHENOL, *o*-, *m*-, AND *p*-CRESOL, *p*-OXYPHENYLACETIC, *p*-OXYPHENYLPROPIONIC, AND *p*-OXYPHENYLLACTIC ACIDS, TYROSINE, AND TYRAMINE.

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A study of the products formed from tyrosine by the action of living cells has always been less difficult than the study of the products formed from histidine because the phenols formed can be easily separated into several well defined groups. Experiments on tyrosine have almost invariably been conducted on a large scale, however, because the products formed have had to be identified and estimated by a process of isolation and purification. A purely chemical method, *applicable to small amounts of material, that would effect a quantitative separation into the maximum possible number of groups and that would permit an accurate determination of the constituents of each group without the necessity for an actual isolation of the constituents,* would be superior to any previously described method.

In the preceding communication,[1] two methods were described by means of which small amounts of phenols can be accurately estimated. In that paper, no claims were made for the applicability of the method to mixtures of phenols. It is the object of this report to show that the methods can also be applied to mixtures of phenols and that the exact quantity of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine can be rapidly and accurately determined. The method has been found to be

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1922, l, 235.

applicable, without modification, to liquid media in which bacteria have been allowed to metabolize tyrosine in the presence of salts and glycerol or glucose. In its present form the method is not directly applicable, in its entirety, to more complex liquids such as urine or blood. We hope to modify the method so that it can be applied to such liquids. The underlying principles of the method are as follows:

The simple phenols that are apt to occur associated with living matter can be divided into four groups.

A. Those volatile with steam (phenol and *p*-cresol).

B. Those that will pass into ether from an acidified aqueous solution (*p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids).

C. Tyramine, which can be extracted from an alkaline (sodium carbonate) solution by means of amyl alcohol.

D. Tyrosine, which remains after all of the other phenols have been extracted and which can be determined colorimetrically if imidazoles and amino-acids are not present in high concentrations.

The acidified mixture of phenols is first subjected to a distillation under ordinary pressures. The volatile phenols, phenol or *p*-cresol,[2] pass over quantitatively into the distillate where they can be determined colorimetrically if only *one* of them is present. If these two phenols are present together in nearly equal amounts our method will not estimate them because phenol gives a yellow and *p*-cresol a red color with alkaline *p*-phenyldiazonium sulfonate; and these two colors cannot be estimated separately with a Duboscq colorimeter.

The residue obtained after the volatile phenols have been removed by distillation, is transferred to a glass dish, concentrated to a syrupy consistency on the water bath, and diluted to exactly 25 cc. Of this solution 10 cc. are transferred to an extraction bottle and extracted ten times with ether using 20 cc. for each extraction. *p*-Oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids pass quantitatively into the ether. The combined ether extracts are treated with water (25 cc.) and phosphoric acid (5 drops of the 85 per cent acid) after which the ether is

[2] *o*- and *m*-cresol are also quantitatively volatile with steam. We have not included them in our analytical process because they are not apt to occur in biological fluids under ordinary conditions.

removed by distillation, at first under ordinary pressures and then *in vacuo*. The aqueous solution is then diluted to 100 cc. and the concentration of aromatic hydroxy-acids determined colorimetrically.

Our method does not differentiate between the members of the aromatic hydroxy-acid fraction if they are present together in the same solution. Usually, however, only one member of this group will be present under any given condition. It is possible to tell not only *how much* of a given acid is present but also *which one of the acids is present* by the character of the color produced. The color produced by *p*-oxphenylacetic acid *develops to its maximum intensity within 2 minutes and is slightly brown.*

The color produced by *p*-oxyphenylpropionic acid is also fully developed within 2 minutes; but it contains no brown and it undergoes a sharp change after 2 to 3 minutes of color constancy, that is characteristic for *p*-oxyphenylpropionic acid. The color, which has matched that of the (CR) comparison standard perfectly, suddenly takes on a cloudy appearance and a bluish tint that makes further comparisons impossible.

The color produced by *p*-oxyphenyllactic acid *comes up slowly so that 5 minutes are required to give a color of maximum intensity.* The color finally obtained is slightly more *yellow* than that of the (CR) comparison standard. It is also quite stable: *it does not fade perceptibly for 5 minutes after it has reached its maximum intensity.* This color does *not* undergo a change like that of *p*-oxyphenylpropionic acid.

The acid-containing aqueous liquid, which has been freed from volatile phenols by distillation and from aromatic hydroxy-acids by ether extractions, is carefully treated with solid anhydrous sodium carbonate until the liquid stops effervescing. An excess of sodium carbonate (2 gm.) is then added and the alkaline aqueous liquid extracted six times with amyl alcohol, using 20 cc. for each extraction. The amyl alcohol extracts contain tyramine and the alkaline aqueous liquid contains tyrosine.

A quintuple extraction of the amyl alcohol extracts with N H_2SO_4 removes the tyramine completely from the amyl alcohol. Tyramine can then be estimated colorimetrically in the aqueous acid liquid after neutralization and dilution to 100 cc.

The tyrosine fraction, which contains an excess of sodium carbonate, is transferred to a glass dish, treated with an excess of 37 per cent HCl, and concentrated on the water bath. The crystalline residue obtained is transferred with water to a 25 cc. graduated cylinder and diluted to the mark. Tyrosine is then estimated colorimetrically, in this fraction.

Sections I to IV of this report contain a detailed account of the results of experiments on known solutions of phenol derivatives by which the accuracy of the technique of the method for separating the phenols, described in Section V was experimentally established.

EXPERIMENTAL PART.

I. Tyrosine and Tyramine Not Destroyed by the Prolonged Action upon Them of Hot Hydrochloric Acid and Sodium Hydroxide.

A. Hot 20 Per Cent Hydrochloric Acid.

Tyrosine.—Tyrosine (0.1000 gm.) was mixed with 100 cc. of 20 per cent HCl in a 400 cc. round bottomed Pyrex flask. The solution was boiled for 24 hours over an electrically heated sand bath. The resulting pale yellow liquid was evaporated on the water bath in a glass dish. The residue was treated with water and sufficient hydrochloric acid to give a clear solution. This liquid was then transferred to a volumetric flask and diluted to 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " " 16.0 " (F−MO).

The entire test liquid must, therefore, have contained 0.1000 gm. of tyrosine which is exactly the amount originally introduced.

Continued boiling with 20 per cent hydrochloric acid does not destroy tyrosine to the slightest extent.

An entirely similar experiment was carried out on 0.1000 gm. of tyramine hydrochloride. Of the solution finally obtained

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " " 16.0 " (F−MO).

This, by table[3] is equal to 0.1000 gm. of tyramine hydrochloride for the entire test solution which is 100 per cent of the amount originally introduced.

Tyramine is not injured to the slightest extent by continued boiling with 20 per cent hydrochloric acid.

B. Hot 10 Per Cent Sodium Hydroxide.

Tyrosine.—Tyrosine (0.10 gm., 10 cc. of a 1 per cent solution) was mixed with 10 cc. of a 20 per cent solution of sodium hydroxide. The resulting solution was then heated for 10 hours on the boiling water bath in a small, long necked, round bottomed flask. The resulting colorless liquid was transferred, with water, to a 1,000 cc. volumetric flask, neutralized to litmus paper with 5 N H_2SO_4, and diluted to 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F—MO) and
0.20 " " " " " " 16.0 " (F—MO),

which for the entire solution represents 0.10 gm. of tyrosine, 100 per cent of the amount originally introduced.

Tyrosine is not injured to the slightest extent when it is heated for 10 hours with 10 per cent sodium hydroxide.

Tyramine.—An entirely similar experiment was carried out on 0.1000 gm. of tyramine hydrochloride. Of the solution finally obtained

0.10 cc. had a color value equivalent to 8.0 mm. (F—MO) and
0.20 " " " " " " 16.0 " (F—MO)

which, by table, is equivalent to 0.10 gm. of tyramine hydrochloride, 100 per cent of the amount originally introduced.

Tyramine is not injured to the slightest extent when it is heated for 10 hours with 10 per cent sodium hydroxide.

II. Phenol and o-, m-, and p-Cresol are Quantitatively Volatile with Steam.

p-Cresol (0.01 gm., 10 cc. of the stock 0.1 per cent solution) was mixed with 140 cc. of water in a 500 cc. distilling flask. The

[3] Tables for converting colorimetric readings into gm. of phenols are given in the preceding article (*J. Biol. Chem.*, 1922, l, 235).

flask was heated with a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral glass condenser, the distillate being collected in a 100 cc. graduated cylinder.

Collections.

1. Total volume of the distillate was 50 cc., of which
0.10 cc. had a color value equivalent to 8.0 mm. (CR).
This, by table, is equivalent to 0.0080 gm. of *p*-cresol, 80 per cent of that originally introduced.
2. Total volume of the distillate was 50 cc. of which
0.40 cc. had a color value equivalent to 6.3 mm. (CR).
This, by table, is equivalent to 0.001575 gm. of *p*-cresol, 15.75 per cent of the amount originally introduced.

At this time, 100 cc. of distilled water were introduced into the distilling flask and the distillation was continued.

3. Total volume of the distillate was 60 cc. of which
1.00 cc. had a color value equivalent to 3.0 mm. (CR).
This, by table, is equivalent to 0.00036 gm. of *p*-cresol, 3.6 per cent of the amount originally introduced.
4. The fourth 50 cc. of distillate had no color value.
The total recovery of *p*-cresol in this case was

First	50 cc. contained	0.0080	gm.
Second	50 " "	0.001575	"
Third	60 " "	0.00036	"
		0.009935	"

which is 99.35 per cent of the amount originally introduced.

A second experiment was now carried out to see if a large quantity of *p*-cresol could be recovered quantitatively. This was a duplicate of Experiment 1 excepting that 0.10 gm. of *p*-cresol was used instead of 0.01 gm.

Collections.

1. Total volume of the distillate was 100 cc. The color produced with 0.10 cc. of this liquid was so intense that a direct comparison was impossible; hence 10 cc. of the distillate were diluted to 100 cc. Of this diluted solution
0.20 cc. had a color value equivalent to 9.4 mm. (CR)
which, by table, is equivalent to 0.094 gm. of *p*-cresol for the entire distillate, 94 per cent of the amount originally introduced.

2. Total volume of the distillate was 100 cc. of which
0.20 cc. had a color value equivalent to 6.3 mm. (CR).
This, by table, is equal to 0.0063 gm. of *p*-cresol for the entire distillate, 6.3 per cent of the amount originally introduced.

3. Total volume of the distillate was 50 cc. of which
1.00 cc. had a color value equivalent to 7.2 mm. (CR).
This, by table, is equal to 0.000072 gm. of *p*-cresol, 0.072 per cent of the amount originally introduced.

4. Total volume of the distillate was 50 cc. of which
1.00 cc. had a color value equivalent to 3.5 mm. (CR).
This is equal to 0.000035 gm. of *p*-cresol, 0.035 per cent of the amount originally introduced.

5. The fifth 50 cc. of distillate had no color value.

The total recovery in this case was

First	100 cc. contained	0.094000 gm.	
Second	100 " "	0.0063 "	
Third	50 " "	0.000072 "	
Fourth	50 " "	0.000035 "	
		0.100407 "	of *p*-cresol

which is 100.4 per cent of the amount originally introduced.

Entirely similar experiments were conducted on solutions of phenol, and *o*- and *m*-cresol. In every case between 99 and 100.5 per cent of the phenol was recovered and accounted for, colorimetrically, in the distillate.

Phenol, o-, m-, and p-cresol are completely volatile with steam and they can be estimated quantitatively in the distillates.

III. The Aromatic Hydroxy-Acids Can Be Quantitatively Extracted from an Acidified Aqueous Solution with Ether.

When an acidified aqueous solution containing any of the aromatic hydroxy-acids is extracted ten times with redistilled ether, the aromatic hydroxy-acids pass quantitatively into the ether because *the aqueous liquid no longer gives the slightest color with Pauly's reagent.*[4] If the ether, which must surely contain all of the aromatic hydroxy-acids, is now removed by distillation, and the residue diluted with water to a definite volume, *a colorimetric determination by the usual process either fails to reveal the presence of any phenol or gives values that are far below the theoretical.*

[4] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

We thought, at first, that the aromatic hydroxy-acids might be slightly volatile with steam or ether vapor; but we soon proved that this was *not* true. The other possibility was that the ether contained some imperfectly volatile substances that prevented the phenol from combining with *p*-phenyldiazonium sulfonate in alkaline solution. We surmised that the interfering substance was an oxidation product of the ether, perhaps peroxide in character. To remove this, we agitated a sample of redistilled ether with alkaline permanganate until the permanganate had been decolorized, and redistilled the ether layer. An extraction of the aromatic hydroxy-acids with this *freshly prepared* ether was then carried out. Theoretical values were always obtained with these ether extracts. If, however, the ether was not used for a day or two, low values were again obtained. To avoid the necessity of having to prepare a fresh supply of ether each day, we modified the usual colorimetric procedure as follows.

(1-X) cc. of water and X cc. of the aromatic hydroxy-acid-containing liquid were treated, for 2 minutes, with 2 cc. of the nitrous acid containing *p*-phenyldiazonium sulfonate reagent. The 1.1 per cent sodium carbonate solution (5 cc.) was then added. This inverse process, which is similar to the process usually used for the qualitative determination of phenols or imidazoles, gave theoretical values both with pure solutions of aromatic hydroxy-acids and with ethereal extracts *even when the ether had not been previously treated with permanganate.* Obviously, then, the free nitrous acid present in the reagent modified the interfering substances so that they no longer prevented the combination between the phenol and the diazonium salt. Fortunately, the nitrous acid did not react with the aromatic hydroxy-acids in such a way as to prevent their coupling with the diazonium salt. *This inverse process should always be used in the estimation of aromatic hydroxy-acids.*

The following experiment will illustrate the method used in the extraction and estimation of the aromatic hydroxy-acids.

p-Oxyphenylpropionic acid (1.00 cc. of the 1 per cent stock solution was mixed in a 35 cc. extraction bottle[5] with 0.2 cc. of 95 per

[5] Any 35 cc. narrow mouthed bottle with a carefully fitted glass stopper will answer the purpose.

cent H_2SO_4 and 9 cc. of water. 20 cc. of specially prepared ether,[6] measured by graduate, were introduced into the bottle, the glass stopper inserted and the liquids vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube and centrifuged for from 1 to 2 minutes. This gave a sharp separation into two layers.

Separation of Ether.—The ether layer was separated from the aqueous layer by means of a device similar to that shown in a previous article.[7] The ether, instead of being drawn into a Squibb funnel, is drawn into a 700 cc. round bottomed flask. Capillary F is, at first, carefully immersed *just below the surface of the ether layer* because the vaporization of the ether as it comes in contact with the large surface of the warm flask usually produces enough pressure to eject some ether back into the extraction bottle which would stir up the aqueous layer if capillary F was too deeply immersed. After the first momentary back pressure, however, capillary F can be gradually lowered until all but a thin film of ether has been drawn into the receiving flask.

The ether not only extracts the aromatic hydroxy-acids but it also reduces the volume of the aqueous layer. It is necessary, therefore, to add sufficient water after each extraction to reestablish the initial volume of 10 cc. It is best to mark the extraction bottle with a carborundum pencil at a level corresponding to a volume of approximately 10 cc.

The above process was repeated nine times so that a total of ten extractions was made.

The aqueous acid liquid gave no Pauly reaction. The ether extracts were then treated with 25 cc. of water and 5 drops of 85 per cent phosphoric acid. The mixture was agitated and subjected to a distillation at first under atmospheric pressure and then *in vacuo* until the ether had been removed entirely. An ebullition tube was *not* used and the distillation was *not* contin-

[6] Commercial ether (800 cc.) is agitated with 50 cc. of alkaline permanganate, such as is used in amino nitrogen determinations by the Van Slyke process, and 50 cc. of water in a 2,000 cc. separatory funnel. After the permanganate has been reduced to MnO_2, the ether layer is poured off and distilled. The redistilled ether so obtained is then ready to use for extractions.

[7] See Fig. 1, Studies on proteinogenous amines. III (Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 526.

ued until all of the water had passed over. The aqueous liquid left in the flask was carefully transferred with water to a 100 cc. graduated precision cylinder and diluted to 100 cc. Of this liquid, 0.10 cc. was mixed with 0.90 cc. of water and 2 cc. of reagent in the right-hand cylinder of the Duboscq colorimeter. After 2 minutes, 5 cc. of the 1.1 per cent sodium carbonate solution were added. The cylinder was transferred to the colorimeter set at 20 mm. and the color compared in the usual manner with that of the (CR) comparison standard. A reading of 3.1 mm. (CR) was obtained. A 0.20 cc. portion of the liquid had a color value of 6.2 mm. This is equal to 0.01 gm. of *p*-oxyphenylpropionic acid, 100 per cent of the amount originally introduced.

Entirely similar experiments were carried out on solutions of *p*-oxyphenylacetic and *p*-oxyphenyllactic acids. 100 per cent recoveries were obtained in every case.

The aromatic hydroxy-acids are quantitatively extracted from an acidified aqueous solution with ether.

IV. Tyramine Quantitatively Separated from Tyrosine by Means of Amyl Alcohol.

A. When a Small Amount of Tyrosine Is Mixed with a Large Amount of Tyramine.

Tyrosine (0.20 cc. of the 1 per cent solution), tyramine (5 cc. of the 1 per cent solution), 0.20 cc. of 95 per cent H_2SO_4, and 5 cc. of water were mixed in a 35 cc. extraction bottle. Anhydrous sodium carbonate was added carefully until the liquid no longer effervesced. Then 2.00 gm. of the carbonate were added and the liquid was agitated until the solid had dissolved. Redistilled amyl alcohol (20 cc., measured by graduate) was introduced into the bottle, the glass stopper inserted, and the liquids were vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube and centrifuged for about 2 minutes. This gave a sharp separation into two layers.

Separation of Amyl Alcohol.—The amyl alcohol layer was separated from the aqueous alkaline layer by means of the device and technique described in a previous article.[7]

The extraction was repeated five times so that a total of 120 cc. of amyl alcohol was used. As in the case of the previously

described ether extractions, the amyl alcohol extracts not only the tyramine but it also markedly reduces the volume of the aqueous layer. It is necessary, therefore, to add sufficient water after each extraction to reestablish the initial volume of 10 cc.

Removal of Tyramine from Amyl Alcohol.—The combined amyl alcohol extracts were extracted five times, in the same Squibb funnel, with 1.0 N H_2SO_4 using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The sulfuric acid extracts were collected in a 100 cc. glass-stoppered precision cylinder and neutralized to litmus paper with 40 per cent sodium hydroxide. The solution was then rendered very faintly acid by adding a few drops of 1.0 N H_2SO_4, transferring to a glass dish, and evaporating on the water bath to remove the amyl alcohol. The crystalline residue was dissolved in water, transferred to a 100 cc. graduated precision cylinder, and diluted to 100 cc. Of the solution so obtained 0.10 cc. had such a high color value that accurate comparisons were impossible; hence 10 cc. of it were diluted with water to 50 cc. Of this diluted solution

0.10 cc. had a color value of 8.0 mm. (F−MO) and
0.20 " " " " " " 16.0 " (F−MO).

This, by table, is equal to 0.05 gm. of tyramine hydrochloride for the entire *original* test solution which is 100 per cent of the amount originally introduced.

The Alkaline Aqueous Liquid (Tyrosine Fraction).—The alkaline aqueous liquid was transferred with water to a glass dish. The dish was covered with a watch-glass. An excess of 37 per cent HCl was added and the liquid concentrated on the water bath. The crystalline residue was dissolved in water with the aid of a few drops of 37 per cent HCl, and diluted to exactly 100 cc. Of this solution

0.50 cc. had a color value equivalent to 8.0 mm. (F−MO) and
1.00 " " " " " " " 16.0 " (F−MO)

which, by table, is equivalent to 0.002 gm. of tyrosine, 100 per cent of the amount originally introduced.

Tyramine is quantitatively extracted from an alkaline (sodium carbonate) aqueous solution by amyl alcohol. Tyrosine, when present in small amounts, does not pass into amyl alcohol from such an alkaline aqueous solution.

B. When a Large Amount of Tyrosine Is Mixed with a Small Amount of Tyramine.

Tyrosine (5.00 cc. of the stock 1 per cent solution), tyramine (0.20 cc. of the stock 1 per cent solution), 0.20 cc. of 95 per cent sulfuric acid, and 5 cc. of water, were mixed in a 35 cc. extraction bottle. Anhydrous sodium carbonate was added carefully until the liquid no longer effervesced. Then 2.00 gm. of the carbonate were added and the mixture agitated until the solid had dissolved. This liquid was then extracted six times with amyl alcohol as described in Section IV, Part A.

Alkaline Aqueous Liquid (Tyrosine Fraction M).—This was treated as described in Section IV, Part A. The solution was finally diluted to 500 cc. of which

0.10 cc. had a color value equivalent to 7.5 mm. (F−MO) and
0.20 " " " " " " 15.0 " (F−MO)

which, by table, is equivalent to 0.0469 gm. of tyrosine. Since 0.0500 gm. of tyrosine had been originally introduced, *0.0031 gm. of tyrosine must have passed into the amyl alcohol.*

First Amyl Alcohol Extract.—The combined amyl alcohol extracts were extracted with N H_2SO_4 as described in Section IV, Part A. It was necessary in this case, as it will be in most cases, to remove the sulfuric acid with barium hydroxide to avoid the accumulation of a large amount of salts which would interfere with the subsequent treatment. The combined acid extracts were transferred to a 250 cc. Pyrex flask and heated on the water bath. Barium hydroxide (9.0 gm.) was dissolved in 50 cc. of hot water. The resulting solution was added slowly to the acid liquid. The faintly *acid* mixture so obtained was digested on the water bath for 2 hours and filtered through a hard folded filter. The paper and contents were thoroughly washed with hot water. The filtrate and washings were collected in a glass dish, exactly neutralized with sodium hydroxide, and evaporated on the water bath. The solid residue so obtained was transferred with the aid of a few drops of 5 N H_2SO_4 and 10 cc. of water to a 35 cc. extraction bottle. The liquid was treated with 2.00 gm. of anhydrous sodium carbonate. This alkaline solution was then extracted with amyl alcohol in the usual manner which again divides the

material into two fractions; the alkaline aqueous liquid II, which should contain the tyrosine that passed into amyl alcohol at the time of the first extraction, and the purified tyramine fraction which should be free from tyrosine.

Alkaline Aqueous Liquid II (Tyrosine Fraction II).—This was acidified, evaporated, and finally diluted to 100 cc. as in the case of the main tyrosine fraction. Of this solution,

0.50 cc. had a color value equivalent to 11.2 mm. (F−MO) and
1.00 " " " " " " " 22.4 " (F−MO)

which, by table, is equivalent to 0.0028 gm. of tyrosine. Since 0.0469 gm. of tyrosine was recovered in the first, main tyrosine fraction, a total of 0.0497 gm. of tyrosine was accounted for. This is 99.4 per cent of the amount originally introduced.

Purified Tyramine Fraction.—This was extracted with N H_2SO_4 as previously described. The acid was nearly neutralized with 40 per cent sodium hydroxide. The resulting *faintly acid* solution was transferred to a glass dish and evaporated on the water bath. The residue was transferred, with water, to a precision cylinder and diluted to 100 cc. Of this solution,

0.50 cc. had a color value equivalent to 7.9 mm. (F−MO) and
1.00 " " " " " " " 15.8 " (F−MO).

This, by table, is equal to 0.001975 gm. of tyramine hydrochloride, 98.75 per cent of the amount originally introduced.

When the concentration of tyrosine is high, a small amount of it passes into amyl alcohol from an alkaline (sodium carbonate) solution. To free the tyramine fraction from this small admixture of tyrosine it is necessary to conduct a second amyl alcohol extraction on the first tyramine fraction. This slightly longer process with its double amyl alcohol extraction is to be recommended because it makes the determination of tyramine certain and reliable. In most cases it is probably advisable to remove the excess of sulfuric acid from the *final* tyramine fraction with baryta because such solutions would then be practically free from salts and ready for physiological experiments.

V. Separation of Phenols into Four Fractions: Volatile Phenols, Aromatic Hydroxy-Acids, Tyramine, and Tyrosine. The Accurate Determination of One Member of Each Fraction.

The method outlined below is primarily intended to be used in bacterial metabolism studies on tyrosine. We have carried out a sufficient number of such metabolism experiments to be certain that the method gives accurate results. We hope to report these experiments in the near future. Some of the steps in the following experiment were taken, not because they were necessary in this case, but because they were necessary in the metabolism experiments.

A solution containing the following was prepared from the stock solutions; 10 cc. of 1 per cent tyrosine, 4 cc. of 1 per cent tyramine hydrochloride, 4 cc. of 1 per cent *p*-oxyphenyllactic acid, 2 cc. of 1 per cent phenol, 80 cc. of water, and 100 cc. of Nutritive Medium 3.[8]

Filtration.—The clear liquid was forced through a Mandler filter.[9] The flask and filter were washed free from phenols with 200 cc. of water. The filtrate was then treated with 0.50 cc. of 95 per cent H_2SO_4.

Estimation of Volatile Phenols (Phenol).—The filtrate and washings were transferred, with water, to a 1,000 cc. long necked, round bottomed, Pyrex flask. The flask was heated with a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral condenser, the distillate being collected in

[8] Nutritive Medium 3 contains:

NH_4Cl	4.00 gm.
KNO_3	2.00 "
KH_2PO_4	8.00 "
NaCl	16.00 "
Na_2SO_4	0.04 "
$NaHCO_3$	8.00 "
$CaCl_2$	0.20 "
Glycerol	80.00 cc.

in a total aqueous volume of 2,000 cc. Koessler, K. K., and Hanke, M. T. *J. Biol. Chem.*, 1919, xxxix, 579.

[9] In bacterial metabolism studies, the hydrogen ion concentration is determined, colorimetrically, on 1 cc. of this filtrate before it is diluted with wash water.

a 250 cc. graduated cylinder. Exactly 200 cc. of distillate were collected of which

0.10 cc. had a color value equivalent to 9.6 mm. (Ph–R) and
0.20 " " " " " " " 19.2 " (Ph–R).

This, by table, is equivalent to 0.01666 gm. of phenol for the entire 200 cc. of test liquid.

The distillation was continued until about 125 cc. of distillate had been collected. This was diluted to exactly 200 cc. Of this solution

0.20 cc. had a color value equivalent to 4.0 mm. (Ph–R) and
0.40 " " " " " " " 8.0 " (Ph–R)

which, by table, is equivalent to 0.0034 gm. of phenol for the entire test solution. In all, 0.02006 gm. of phenol was obtained which is 100.3 per cent of the amount originally introduced. The colors obtained were, in every case, exactly like that produced by pure phenol; hence none of the other phenols volatilized.

Estimation of Aromatic Hydroxy-Acids (p-Oxyphenyllactic Acid).— The liquid left in the flask was carefully transferred, with water, to a glass dish and concentrated on the water bath. The pale yellow syrup was transferred, with water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. We will refer to this as the *test liquid.*

Of this acid test liquid, exactly 10 cc. (measured by pipette) were transferred to a 35 cc. extraction bottle and extracted ten times with specially prepared ether as described in Section III of this paper.

The ether extracts were then treated as described in Section III. The solution finally obtained (volume 100 cc.) was examined colorimetrically for *p*-oxyphenyllactic acid.

0.10 cc. had a color value equivalent to 4.0 mm. (CR)
0.20 " " " " " " " 8.0 " (CR)
0.30 " " " " " " " 12.0 " (CR).

The colors were exactly like that obtained with a pure solution of *p*-oxyphenyllactic acid. This, by table, is equivalent to 0.0400 gm. of *p*-oxyphenyllactic acid *for the entire* 25 cc. *of test liquid*, which is 100 per cent of the amount originally introduced.

Separation of Tyramine from Tyrosine.—The acid liquid left in the extraction bottle, equivalent to 10 cc. of the test liquid, was carefully treated with anhydrous sodium carbonate until the liquid no longer effervesced. Then 2 gm. of the carbonate were added. The mixture was warmed and agitated until the solid had passed into solution. This solution was then extracted six times with amyl alcohol as described in Section IV of this paper.

The Alkaline Aqueous Liquid (Tyrosine Fraction M).—The alkaline liquid left in the extraction bottle was transferred to a glass dish with 100 cc. of water. The liquid was concentrated on the water bath to a volume of about 50 cc., which removed the ammonia completely. The liquid was then treated with 3.5 cc. of 37 per cent HCl, precautions being taken to prevent loss of the solution through spattering. The strongly acid liquid was concentrated on the water bath. The crystalline residue was dissolved in water, with the aid of a few drops of 37 per cent HCl, transferred to a graduated precision cylinder, and diluted to 25 cc. This is the main tyrosine fraction M.

Of this solution 5 cc. (measured by pipette) were diluted to 80 cc. Of this diluted solution

0.10 cc. had a color value equivalent to 7.3 mm. (F–MO) and
0.20 " " " " " " " 14.6 " (F–MO)

which, by table, is equivalent to 0.0912 gm. of tyrosine for the entire original test liquid, 91.2 per cent of the amount originally introduced. As one would expect from Experiment B, Section IV, some tyrosine passed into the amyl alcohol from which it will be recovered when the second extraction is carried out (see below).

Amino Nitrogen Determination on Tyrosine Fraction M.—The above tyrosine fraction M (5 cc.) was subjected to an amino nitrogen determination by the Van Slyke method. 1.07 cc. of N_2 were obtained at 25° and 747 mm. which is equal to 0.00729 gm. of nitrogen for the entire test liquid. Tyrosine always gives off about 2 per cent more gas by the Van Slyke process than it should theoretically. We have found this to be invariably true and Van Slyke[10] gives figures that are in perfect agreement with

[10] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 193.

this statement. This nitrogen figure must, therefore, be reduced by 2 per cent before the tyrosine value is calculated. The corrected nitrogen figure is 0.00715 gm.; which is equivalent to 0.00925 gm. of tyrosine, 92.5 per cent of the amount originally introduced. The check between the tyrosine values obtained by these two methods is good.

We wish again to call attention to the fact that this fraction *does not* contain *all* of the tyrosine. A second fraction is obtained later when the first tyramine fraction is reextracted with amyl alcohol. The second tyrosine fraction so obtained usually contains about 0.003 gm. of tyrosine which is too little to estimate by the amino nitrogen method but which is easily determined colorimetrically.

First Amyl Alcohol Extract (Tyramine Fraction I).—This fraction contains all the tyramine together with a small amount of tyrosine. A quantitative separation can be effected by carrying out a second extraction with amyl alcohol in alkaline solution.

The amyl alcohol was, therefore, extracted with N H_2SO_4, the acid removed with baryta, the resulting liquid made alkaline with sodium carbonate and reextracted with amyl alcohol as described in Section IV, Part B.

Second Alkaline Aqueous Liquid (Tyrosine Fraction II).—This was acidified, evaporated, and finally diluted to 100 cc. as described in Section IV, Part B. In this case the ammonia was not removed by evaporation because an amino nitrogen determination is not to be carried out. Of the solution so obtained

0.20 cc. had a color value equivalent to 4.8 mm. (F−MO) and
0.40 " " " " " " " 9.6 " (F−MO)

which, by table, is equal to 0.0075 gm. of tyrosine for the *entire original test liquid.* Since 0.0912 gm. of tyrosine was recovered in the main tyrosine fraction M, a total of 0.0987 gm. of tyrosine was accounted for, which is 98.7 per cent of the amount originally introduced.

Purified Tyramine Fraction (Second Amyl Alcohol Extract).—This was extracted six times with N H_2SO_4 as previously described. The acid was nearly neutralized with baryta, the barium sulfate removed by filtration, the filtrate concentrated on the water bath in a glass dish, and the residue dissolved in water and diluted to

exactly 100 cc. as described in Section IV, Part B.[11] Of this solution

0.10 cc. had a color value equivalent to 12.8 mm. (F−MO)

which, by table, is equal to 0.0400 gm. of tyramine hydrochloride, 100 per cent of the amount originally introduced.

SUMMARY.

This paper contains the description of a method by means of which volatile phenols, aromatic hydroxy-acids, tyramine, and tyrosine can be quantitatively separated and estimated. The phenols are determined by a colorimetric process described in the preceding paper. Volatile phenols—phenol, *o*-, *m*-, and *p*-cresol—are distilled off and estimated in the distillate. Aromatic hydroxy-acids—*p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids—are extracted with ether from the acidified aqueous liquid which has been freed from volatile phenols by distillation. The aromatic hydroxy-acids are estimated in the ether extracts. The remaining liquid, which contains all of the tyramine and tyrosine, is made alkaline with sodium carbonate and freed from tyramine by extraction with amyl alcohol. Tyramine is then determined in the amyl alcohol extract; tyrosine is determined in the alkaline aqueous liquid. The separations are quantitative and the colorimetric determinations are accurate to 0.5 to 1.5 per cent.

[11] It is necessary to remove the excess of H_2SO_4 with baryta only if physiological or isolation experiments are to be carried out on this fraction. In other cases it is simpler to neutralize with 40 per cent NaOH as described in Section IV of this report.

SOLUBILITY OF CARBON MONOXIDE IN SERUM AND PLASMA.*

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INTRODUCTION.

In the course of the work of the writers on a method of determination of carbon monoxide in blood, the question arose as to how much of the total gas united with the hemoglobin, and how much merely dissolved in the serum. With the known strong affinity of carbon monoxide for hemoglobin (220 to 300 times as strong as that of oxygen) (1) it would be expected that by far the largest percentage would enter into combination with the hemoglobin, but it seemed of value to investigate just what percentage could be accounted for as being in simple solution. This was especially the case with the Van Slyke method, where the total amount of carbon monoxide in the blood is measured, and not the amount of carbon monoxide hemoglobin. Van Slyke (2) passes over the subject in the case of carbon monoxide; but when dealing with oxygen in the blood (3) has a table of deductions and corrections.

These are estimated on the basis of Bohr's recommendation that the solubility of air in serum is roughly nine-tenths that in water at the corresponding temperature.

On this subject of the solubility of gases in serum and blood, Bohr goes quite into detail (4). He states[1] that the absorption coefficients of oxygen and carbon dioxide in whole blood and that of carbon dioxide in plasma cannot be obtained directly,

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[1] Bohr (4), p. 62.

because there is also a chemical reaction taking place. But the percentage of deduction (or relation of the coefficient in plasma to that in water) of various gases, when they do not react, is about the same (5). Therefore, when he made determinations and found that the absorption coefficients of oxygen and nitrogen in plasma are 97.5 per cent of those in water, and the absorption coefficient of hydrogen in whole blood is 92 per cent of the figures in water, he felt safe in making the generalization. For later comparison his figures are given; they are calculated by the aid of his deductions.

Coefficient of Absorption in Cc. of Gas (at 0° and 760 Mm.)

	O_2		N_2		CO_2	
	15°	38°	15°	38°	15°	38°
Blood plasma	0.033	0.023	0.017	0.012	0.994	0.541
Whole blood	0.031	0.022	0.016	0.011	0.937	0.511
Blood corpuscles	0.025	0.019	0.014	0.010	0.825	0.450

In the case of carbon monoxide, he says[2] simply that the plasma absorbs the gas physically, but proportionately to the tension, and in a slightly lesser amount than would be absorbed by the same volume of water. It seemed worth while, therefore, to make direct determinations instead of relying on estimates. During the progress of this work, conducted at the Pittsburgh station of the Bureau of Mines, some other data were obtained which resulted in certain important conclusions on the use of a table of deductions to correct for the amount of carbon monoxide in the blood, uncombined with the hemoglobin.

Method of Obtaining Serum and Plasma.

As a medium of investigation, beef serum was selected as the one most readily obtainable in quantity; furthermore, most of the related work was being done on beef blood. To supplement this the results were checked on sheep and human sera. Van Slyke and others have assumed that the solubility of carbon monoxide is the same in serum and in plasma. This was also checked

[2] Bohr (5), p. 122.

on beef plasma. The serum in each case was gotten by allowing the blood gathered in the slaughter house to clot quietly in sterile containers, pouring off the serum, and removing remaining corpuscles with a high speed centrifuge running for 10 to 20 minutes. Beef serum has a golden tinge; that of sheep blood is grayer. With human blood, considerable trouble arose from a tendency on the part of the corpuscles to hemolyze; but a few specimens of good serum were obtained. Beef plasma was gotten from blood caught in a sterile container as it poured from the vessels of a freshly killed animal. This was at once poured into 100 cc. bottles containing as a preservative, 0.2 gm. of sodium oxalate, 0.3 gm. of fluoride, and 0.4 gm. of citrate, well mixed. The blood was centrifuged for 3 to 4 hours; the supernatant plasma then pipetted off and further purified by half an hour more in the centrifuge. The oxalated blood separates most readily; best results are gotten by centrifuging within 2 or 3 days after it is drawn from the animal.

Introduction of Carbon Monoxide into the Liquid.

The carbon monoxide used was made by dropping formic acid into concentrated sulfuric acid at 150°C. The evolved gas was washed through potassium hydroxide and stored in a gasometer over water, whence it was delivered as needed, by water displacement. The specimen of serum was allowed to reach thermal equilibrium within a thermostat adjusted to the desired temperature. The carbon monoxide reached this same temperature by being forced through a glass spiral also placed within the thermostat. From here it passed through a bubbler into the serum. 15 minutes were thought sufficient for the saturation of the sample. Frothing was prevented within the specimen tube by the addition of a drop of caprylic alcohol. The effluent gas passed off through a tube into a hood; the serum was thus saturated at atmospheric pressure.

The analysis of the gases dissolved in the serum was performed on the Van Slyke apparatus. All sera saturated below room temperature were kept in ice water, to prevent loss of gas, pending the time when analysis could be made. The method of analysis employed was a modification of that used by Van Slyke in his

determination of oxygen, hemoglobin, and carbon monoxide in blood. It consists in general of drawing off the gases under a vacuum produced by a mercury pump, and of analysis of the evolved substances. The exact technique used in the Bureau laboratory is as follows.

Technique of the Analysis of Gases Dissolved in Blood Serum by the Van Slyke Apparatus.

Before each analysis the apparatus is washed out first with a solution of concentrated ammonia diluted with 3 volumes of water and then twice with distilled water.

5 cc. of serum are measured by pipette into the cup. The end of the pipette is held below the surface of the liquid after starting the delivery, so as to reduce as much as possible exposure of the liquid surface to air. 2 drops of caprylic alcohol are added to the serum, the liquid is drawn into the burette, and the capillary above the stop-cock is sealed with mercury. The apparatus is evacuated and shaken for 2 minutes. The serum is drawn down into the lower bulb and the extracted gases are measured over mercury at atmospheric pressure as described by Van Slyke. The serum is run back into the extraction chamber and shaken further for 1 minute under the same conditions, then the gas volume is read as before. The extraction is repeated until the volume is constant. It is recorded as total gases. The volume contracts a little with standing only a few seconds, as CO_2 dissolved in the small amount of serum on top of the mercury. Oxygen, CO, and N_2 are completely given off by 1 minute shaking in vacuum; the CO_2 comes off much more slowly, requiring usually about 2 minutes.

0.5 cc. of 10 per cent KOH is added to the cup, and is carefully drawn into the pipette, the mercury in the leveling bulb being held slightly below that in the burette. The CO_2 is quickly absorbed. The volume of contraction is noted and the KOH is drawn down into the lower bulb with the serum.

About 5 cc. of potassium pyrogallate[3] solution are next put into the cup and a drop of straw oil quickly added on top of the pyro

[3] 120 gm. of KOH are added to 80 cc. of water, and 50 gm. of pyrogallic acid to 150 cc. of water; 300 cc. of the alkali are then mixed with 40 cc. of the acid solution.

to exclude the oxygen of the air. The "pyro" is drawn into the burette, but not the straw oil, as the latter interferes with gas absorption by solutions, especially CO by Cu_2Cl_2. Oxygen absorption is slow, but is hastened by working the leveling bulb down and up to insure complete contact of gas with the "pyro." When the volume has become constant the "pyro" is drawn down into the liquid in the lower bulb. The volume of gas is read as before. The cup is rinsed out with distilled water, as the pyrogallate left would form a precipitate with the next reagent to be used. A small dropping pipette has been found convenient for removing liquids from the cup.

0.5 cc. of ammoniacal cuprous chloride[4] is now added and carefully drawn through the remaining gases. CO is quickly absorbed and a constant volume obtained almost immediately. The remaining gas is probably nitrogen, though its volume is sometimes higher than would be expected. A complete analysis is made by this method in less than 30 minutes. With practice, readings may easily be made to within 0.005 cc.

In checking up the carbon monoxide determination by this method, the total gases extracted from untreated serum were analyzed as a blank. An analysis made on sheep serum will serve as an example of the results that were obtained.

Volume of total gas.			Volume after absorption by		
1st extraction.	2nd extraction.	3rd extraction.	KOH	Pyro.	Cu_2Cl_2
cc.	cc.	cc.	cc.	cc.	cc.
0.270	0.290	0.280	0.065	0.060	0.060

It was found that ammoniacal cuprous chloride does not give contraction in volume of the gases extracted from serum when CO gas is not present.

[4] 400 gm. of cuprous chloride and 500 gm. of NH_4Cl are dissolved in 1,500 cc. of water. For use this is mixed with NH_4OH (sp. gr. 0.90) in proportions of 3:1 (Winkler, L. W., Handbook of technical gas analysis, London, 2nd English edition, 1902, 73).

TABLE I.

Solubility of 100 Per Cent CO in Serum and Plasma in Cc. of Gas per Cc. of Serum.

Temperature.	Beef serum.	Sheep serum.	Human serum.	Beef plasma.
°C.				
15	0.0203	0.0210	0.0209	0.0203
	0.0203	0.0201		0.0195
	0.0203	0.0206		0.0198
20	0.0185	0.0183		0.0181
	0.0176	0.0191	0.0180	0.0181
	0.0181	0.0187		0.0181
25		0.0148		
	0.0157	0.0148	0.0183	0.0173
	0.0166	0.0173	0.0183	0.0164
	0.0161	0.0156	0.0183	0.0169
30		0.0150		
		0.0141		
		0.0158		
	0.0150	0.0169		0.0147*
	0.0140	0.0150	0.0158	0.0147
	0.0145	0.0153		0.0147
37				0.0131
	0.0136			0.0150
	0.0117	0.0153		0.0127
	0.0136	0.0144	0.0143	0.0131
	0.0126	0.0135	0.0142	0.0131
	0.0129	0.0144	0.0142	0.0134

* A sample is shown to illustrate the method of calculating Table I.

0.080 cc. of CO was gotten from a 5 cc. sample, saturated at 30°C., and analyzed at 27°C. and 742 mm.

0.016 = CO per cc. of sample.

$$0.016 \times \frac{273}{300} \times \frac{745 - 22}{760} \times \frac{760}{745 - 32} = 0.0147$$

The first fraction corrects for temperature, the second for the partial pressure of the analyzed gas (the barometric pressure minus water vapor tension at 27°), and the third for the partial pressure under which the gas went into solution (barometric pressure less vapor tension of serum at 30°). There are no figures in the literature for this last, but it is taken as approximately equal to that of water. As a change of 10 mm. in pressure alters the result only 0.0002, which is less than the possible error in reading the apparatus, this approximation seems reasonable.

Determination of Solubility of Carbon Monoxide.

As a satisfactory method has now been developed, a series of determinations was carried out at five different temperatures with the four fluids; *i.e.*, beef, sheep, and human sera, and beef plasma. The figures obtained are shown in Table I.

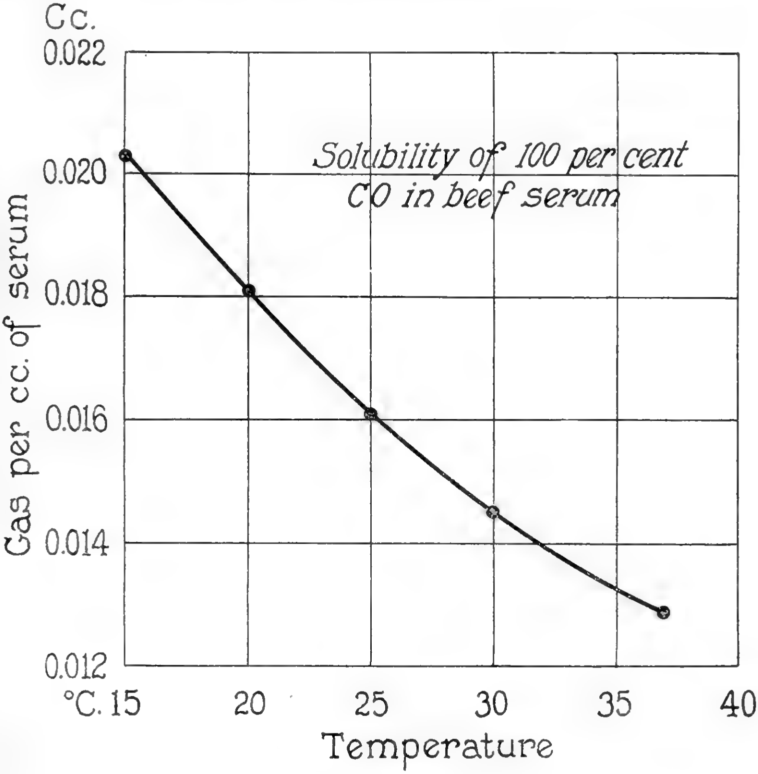

CHART 1.

DISCUSSION.

As would be expected, the figures for the three different sera are practically identical. It is especially interesting to find the results the same with serum and plasma. The accompanying comparison with the table of solubility of carbon monoxide in water (Winkler) gives figures of the same magnitude, but not

quite agreeing with Bohr's hypothesis of a 9:10 relation. In fact, the ratio swings from 8:10 to 7:10 as the temperature rises.

As a check on our method, the same procedure was used in making a determination of the solubility of carbon monoxide in water. Before being used, the water was doubly distilled, then freshly boiled. It was saturated with CO, and then analyzed in the way described above for serum. The results obtained (shown in Table II) are quite in agreement with the figures of Winkler.

TABLE II.

Temperature.	Solubility of 100 per cent CO in beef serum and water.			
	1	2	3	4
	CO per cc. of serum.	CO per 1 cc. water.		Column 1 ÷ Column 3.
		Winkler.*	Author's.	
°*C.*	*cc.*	*cc.*	*cc.*	
15	0.0203	0.02543	0.0253	0.80
20	0.0181	0.02319	0.0236	0.77
25	0.0161	0.02142	0.0213	0.76
30	0.0145	0.01998	0.0198	0.73
35		0.01877		
37	0.0129		0.0179	0.72
40		0.01775		

* Solubility of CO in water (Winkler, L. W., *Ber. chem. Ges.*, 1901, xxxiv, 1408; *Z. physik. Chem.*, 1892, ix, 171.

The application of these figures of solubility to the calculation of results from the Van Slyke method for the determination of CO and hemoglobin in blood presented some difficulty. As carbon monoxide passes from air into blood, the reaction may be pictured somewhat as follows:

$$\begin{array}{l} \text{CO (in air)} \\ \uparrow\downarrow \\ \text{CO (dissolved)} \rightleftharpoons \text{CO-hemoglobin} \end{array}$$

When the hemoglobin is saturated it might be assumed that the serum is also, and the figures of solubility might be subtracted from the total results. But when the hemoglobin is not entirely saturated with carbon monoxide, is a saturated condition of the

serum to be expected? In view of the strong affinity of the gas for hemoglobin, it would be safe to assume that the tendency in the equation above is to the right. Other and similar questions arise. It may be mentioned that the air in cases of fatal carbon monoxide poisoning rarely contains more than 5 to 10 per cent of the gas (frequently not more than 1 to 2 per cent). From the laws of partial pressure of gases in mixtures, it would be expected that the volume of any one constituent in solution would be proportional to its tension and solubility coefficient in the gaseous mixture with which the solution is saturated.

Saturation of Serum with Carbon Monoxide Mixed with Air.

To ascertain whether this expectation is realized in the case of carbon monoxide in serum, beef serum was saturated at 15 and 37°C., with air which contained 1.13 per cent carbon monoxide (analyses by Bureau gas laboratory), with the results shown in Table III.

The results indicate clearly that the usual laws of partial pressures in gaseous mixtures apply in the case of carbon monoxide in serum, and, therefore, in plasma. The volume of the gas dissolved in 5 cc. of serum, even in as rich a mixture as 10 per cent, is so slight (0.010 cc.) that the experimental error in handling the Van Slyke apparatus with a blood sample would equal or exceed the figure. There is, therefore, in ordinary conditions of CO poisoning, no advantage to be gained in accuracy by subtracting from the total result any figure allowed for carbon monoxide dissolved in serum.

The values obtained are also of importance in their bearing on another question. The figures given by Bohr, cited earlier in this paper in regard to the solubility of gases in plasma and in water, are fundamental in the calculations of hydrogen ion concentrations. In the case of carbon monoxide we have found absorption coefficients whose ratios differ sharply from the 9:10 one Bohr assumed. It seems possible, therefore, that the results of this work may be found to have some influence on the basic figures for hydrogen ion determinations.

TABLE III.

Serum saturated at temperature.	Total gas.	CO_2	O_2	N_2	CO
°C.	cc.	cc.	cc.	cc.	cc.
15	0.135	0.040	0.035	0.060	
	0.120	0.035	0.020	0.055	
37	0.250	0.175	0.025	0.050	
	0.195	0.125	0.020	0.050	

The experiment was repeated with air having 9.8 per cent carbon monoxide (Table IV).

TABLE IV.

Serum saturated at temperature.	Total gas.	CO_2	O_2	N_2	CO
°C.	cc.	cc.	cc.	cc.	cc.
15	0.195	0.090	0.025	0.070	0.010
	0.165	0.060	0.030	0.065	0.010
37	0.120	0.040	0.020	0.050	0.010
	0.110	0.045	0.020	0.045	0.010

The results of the second experiment may be expressed in percentage of the total gas (Table V).

TABLE V.

Specimen.	Per cent of total gas* as:			
	CO_2	O_2	N_2	CO
Air.		18.9	71.3	9.8
Serum at 15°.		23.8	66.7	9.5
		28.6	61.9	9.8
Serum at 37°.		25.0	62.5	12.5
		26.7	60.0	13.8

* Carbon dioxide was disregarded in figuring percentages, as it was absent in the original air bubbled through the serum (although of course from the dissolved CO a small amount of free gas would be found above the solution).

CONCLUSIONS.

The writers have devised a method for the determination of carbon monoxide in serum and plasma. Beef, sheep, and human sera, and beef plasma were saturated with the gas at 15, 20, 25, 30, and 37°C. Compatible figures were obtained. These were of the same magnitude as those of the known solubility of carbon monoxide in water, but only about three-fourths as large as the latter. The method was checked by a determination of the solubility of carbon monoxide in distilled water. The solubility figures in serum and plasma are identical.

Further work was done on the solubility of carbon monoxide by exposing the sera and plasma to mixtures (1 to 10 per cent) of carbon monoxide in air. The amount of this gas dissolved under those conditions was so very small that in calculating results in cases of poisoning under ordinary conditions, no allowance need be made for carbon monoxide dissolved in the serum. The results obtained may have some bearing on hydrogen ion calculations.

In the course of the work the writers have received valuable suggestions from several laboratory and clinical men. They wish especially to express their appreciation for the assistance of Dr. R. R. Sayers, Chief Surgeon of the Bureau of Mines, and A. C. Fieldner, Superintendent of the Pittsburgh Experiment Station who supervised the work; Dr. N. R. Givens of the laboratory staff of the West Penn Hospital, Pittsburgh, for advice in purifying serum and plasma; Mr. Dan Monahan of the Pittsburgh Provision and Packing Company, who very kindly furnished beef and sheep blood for quantity work; Dr. J. C. Burt, who placed the facilities of the state clinic at the disposal of the writers in gathering specimens of human blood; Dr. Stegeman, Professor of Physical Chemistry at the University of Pittsburgh, for advice in connection with several points, and to Dr. D. D. Van Slyke of The Rockefeller Institute, and Dr. Yandell Henderson and Dr. H. W. Haggard of Yale University for suggestions and criticisms.

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THE DETERMINATION OF SODIUM IN SERUM WITHOUT THE USE OF PLATINUM DISHES.

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A few months ago Kramer and Tisdall reported from this laboratory "A simple method for the direct quantitative determination of sodium in small amounts of serum."[1] In this method the sodium is precipitated in platinum dishes. Most clinical laboratories do not possess such dishes. An attempt was, therefore, made to see whether equally good results could not be obtained with dishes of less expensive material.

TABLE I.

Sodium Determinations in Platinum and in Tin Dishes.

Sample.	Sodium per 100 cc. of serum.		Diagnosis.
	Platinum.	Tin.	
	mg.	*mg.*	
Solution "B"	350	347	Known solution; theory, 350 mg.
501	320	325	Normal adult.
523	333	330	" "
"C"	328	326	Epilepsy, adult.
"H"	343	338	Normal adult.
601	320	300	" child.
603	291	295	Scurvy, adult.
"S"	304	309	Rickets.

Four dishes were purchased at a 5 and 10 Cent Store. These are sold in groups of four and are intended for making corn muffins. They are about 9 cm. in diameter and about 3 cm. deep. Parallel determinations on serum were made with these and with platinum

[1] Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvi, 467.

dishes. The results are given in Table I. Samples of a solution of "blood salts" containing known amounts of sodium were also analyzed. The agreement was satisfactory.

CONCLUSIONS.

1. Sodium determinations on serum may be done without platinum dishes.
2. Results obtained when such determinations are done in so called "tin dishes" and in platinum show satisfactory agreement.

THE METABOLISM OF SULFUR.

IV. THE OXIDATION OF CYSTINE IN THE ANIMAL ORGANISM.

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(Received for publication, December 13, 1921.)

The mechanism of the intermediary metabolism of the more complex amino-acids still remains obscure. It is generally conceded that, as far as concerns the α-amino group, deamination is accompanied by oxidation with the formation of α-keto or α-hydroxy derivatives of the amino-acids. While the manner of oxidation of the cyclic portions of such amino-acids as contain the benzene or other rings is not fully known, it is usually considered that their oxidation is involved in the further oxidation of the keto- or hydroxy-acids, products of deamination. Thus, phenylalanine in moderate amounts is readily and completely oxidized in the body, while phenylpropionic acid (1) is oxidized only to benzoic and cinnamic acids, and eliminated in conjugation with glycocoll without further oxidation. Phenyllactic and phenylpyruvic acids (2), both products of the normal deamination of phenylalanine, are completely oxidized, while β-phenylalanine (3, 4) and phenylserine (3), and their respective deamination products, β-hydroxyphenylpropionic acid and phenylglyceric acid (3) undergo no oxidation of the benzene ring.

Our knowledge of the normal path of oxidation of the sulfur-containing amino-acid, cystine, is even more hazy. The conversion of cystine through cysteinic acid to taurine *in vitro* has been effected by Friedmann (5). However, even if it is to be considered as definitely proved that taurine originates *in vivo* from cystine (6, 7, 8), the fact that the sulfur in taurine itself is oxidized to sulfates with difficulty, if at all (9, 10, 11), in the animal body, makes it improbable that taurine is primarily concerned in the intermediary metabolism of cystine.

The present investigation represents an attempt to determine some of the factors which are involved in the oxidation of the sulfur of the cystine molecule in the animal organism. The methods used in the study of the oxidation of the aromatic amino-acids already discussed, have included a study of the behavior of the products of deamination of the amino-acids in the body. Desaminocystine (12), a derivative of this type, has been prepared, but does not lend itself readily to experiments of the sort undertaken in the present series. We have, therefore, studied the oxidation of the sulfur fraction of the molecule under conditions which prevented deamination of the cystine in order to determine whether, as is probably the case with those amino-acids, which contain the benzene ring, the possibility of oxidation is connected with the cleavage of the amino group and the oxidation of the keto or hydroxy derivatives.

The phenyluramino derivative of cystine has been prepared and its behavior studied in the organism of the rabbit. It has already been shown that uramino-acids are stable in the animal body and do not yield their nitrogen as urea. Salkowski (13) fed the sodium salt of hydantoic acid (uramino derivative of glycocoll) to rabbits and recovered the acid from the urine in a large measure unchanged. Lewis (14) administered the ethyl ester of the same uramino-acid to rabbits and a dog. In no case was there observed an increased elimination of urea although the nitrogen administered was eliminated in the urine. By the preparation of a derivative from the urine evidence was obtained of the presence of the unchanged ester. Rohde (15) was able to recover the uramino derivation of active leucine from the urine of a cat after intravenous injection.

EXPERIMENTAL.

Phenyluraminocystine was prepared from cystine (obtained by hydrolysis of human hair) and phenylisocyanate according to the method of Patten (16). The recrystallized product showed on analysis results which corresponded closely to the theoretical values for sulfur and nitrogen.

The animals used were in all cases rabbits, which were maintained on a diet of milk to which cane-sugar was added to increase the calorific value. Oats were also added in some cases. The

phenyluraminocystine was suspended in water and sufficient sodium carbonate or hydroxide added to effect solution. This solution was either injected subcutaneously or fed through a gastric sound with a portion of the day's allowance of milk. In one experiment (Table V) the phenyluraminocystine was suspended in milk and fed in this form. Cystine was administered either as the sodium salt or as the hydrochloride. No toxic symptoms were noted in any case.

The bladder was emptied by gentle pressure at the same hour daily and the urine thus obtained added to the urine collected from the cage. Total sulfate sulfur was determined according to

TABLE I.

Rabbit A. Male, black. Weight 2.77 kilos. Daily diet: 200 cc. of milk and 10 gm. of cane-sugar.

Date.	N	Total S.	Total SO_4 S.	Unoxidized S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
Oct. 17	1.136	0.057	0.043	0.014	75.4	24.6	
" 18	0.848	0.036	0.029	0.007	80.6	19.4	
" 19	0.913	0.043	0.035	0.008	81.4	18.6	1.0 gm. phenyluraminocystine *per os* (S = 0.134 gm.).
" 20	1.056	**0.121**	**0.055**	**0.066**	**45.5**	**54.5**	
" 21	0.997	0.057	0.035	0.022	61.4	38.6	
" 22	0.960	0.041	0.035	0.006	84.3	15.7	
" 23	0.943	0.037	0.031	0.006	83.8	16.2	
" 24	1.050	0.048	0.039	0.009	81.3	18.7	1.0 gm. phenyluraminocystine *per os* (S = 0.134 gm.).
" 25	1.055	**0.129**	**0.059**	**0.070**	**45.7**	**54.3**	
" 26	1.006	0.069	0.043	0.023	62.3	37.7	
" 27	1.320	0.073	0.059	0.014	80.8	19.2	
" 28	1.158	0.062	0.053	0.009	86.9	13.1	

Folin; total sulfur by Miss Denis' modification of the Benedict method. Unoxidized sulfur was determined by difference.

The results are presented in Tables I to V. When the sodium salt of phenyluraminocystine was administered *per os* (Tables I and II), there was observed a slight rise in the elimination of total sulfate sulfur, corresponding roughly to about 20 per cent of the sulfur fed in most cases. The greater part of the sulfur of the complex was, however, recovered in the urine as unoxidized sulfur. There was always noted a delayed elimination of part of this "extra" unoxidized sulfur on the day after the administration of the compound. In the first experiment recorded in Table I,

the average daily eliminations of total sulfur and sulfate sulfur during the fore and after periods were 0.040 and 0.033 gm. respectively. During the experimental day and the following day the excretions of total sulfur and sulfate sulfur were 0.178 and 0.090 gm. respectively. This corresponds to the elimination of 0.098 gm. of "extra" total sulfur, of 0.024 gm. of "extra" sulfate sulfur, and of 0.074 gm. of "extra" neutral sulfur. It might be argued that the rise in sulfate sulfur was occasioned by an increased destruction of tissue, resulting from the toxic action of the phenylur-

TABLE II.

Rabbit B. Male, white. Weight 2.2 kilos. Daily diet: 150 cc. of milk and 10 gm. of cane-sugar.

Date.	Total S.	Total SO_4 S.	Unoxidized S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
Nov. 9	0.026	0.018	0.008	69.2	30.8	
" 10	0.022	0.012	0.010	54.5	45.4	
" 11	0.023	0.013	0.010	58.7	41.3	
" 12	**0.110**	**0.091**	**0.019**	**82.7**	**17.3**	0.501 gm. cystine as sodium salt *per os* (S = 0.134 gm.).
" 13	0.042	0.034	0.008	80.9	19.1	
" 14	0.025	0.018	0.007	72.0	28.0	
" 15	**0.115**	**0.038**	**0.077**	**33.0**	**67.0**	1.0 gm. phenyluraminocystine *per os* (S = 0.134 gm.).
" 16	0.047	0.021	0.026	44.7	55.3	
" 17	0.033	0.018	0.015	54.5	45.5	
" 18	0.038	0.028	0.010	73.9	26.1	
" 19	0.028	0.021	0.007	75.0	25.0	
" 20	**0.132**	**0.041**	**0.091**	**31.1**	**68.9**	0.5 gm. phenyluraminocystine at 10.30 a.m. *per os* and same dose at 2.30 p.m. (S = 0.134 gm.).
" 21	0.072	0.048	0.024	66.7	33.3	
" 22	0.036	0.028	0.008	77.8	22.2	
" 23	0.037	0.030	0.007	81.1	18.9	

aminocystine. The excretion of such an amount of sulfate sulfur from this source should be accompanied by increased nitrogen elimination, which on the basis of a N : S ratio in protein of 14 would amount to over 0.300 gm. However, no appreciable change in the nitrogen excretion was observed. It must be concluded that a partial oxidation of the sulfur has occurred. The total sulfur recovered corresponded to 73.1 per cent of the intake. The detailed figures for the other experiments are very similar to the ones just discussed. In the experiments recorded in Table II,

cystine was fed to afford a control experiment and to demonstrate that the sulfur of cystine administered appeared promptly in the urine as sulfate sulfur. It seemed possible that the slight oxidation of phenyluraminocystine which was observed might be the result of bacterial action in the intestine and that if absorption was facilitated the increase in sulfate sulfur might be lessened. To test out this point the phenyluraminocystine was administered in two doses in order to provide increased opportunity for absorption (Table II). No differences in the degree of oxidation were noted.

TABLE III.

Rabbit C. Male, red. Weight 3.64 kilos. Daily diet: 40 gm. of oats and 150 cc. of milk.

Date.		N	Total S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920		*gm.*	*gm.*	*gm.*	*gm.*	
Dec.	4	1.13	0.067	0.049	0.018	
"	5	1.44	0.082	0.060	0.022	
"	6	1.33	0.069	0.053	0.016	
"	7	1.32	0.078	0.062	0.016	1.0 gm. phenyluraminocystine subcutaneously (S = 0.134 gm.).
"	8	**1.50**	**0.154**	**0.060**	**0.094**	
"	9	1.48	0.062	0.040	0.022	
"	10	1.49	0.078	0.061	0.017	
"	11	1.52	0.098	0.080	0.018	0.501 gm. cystine as sodium salt subcutaneously.
"	12	**1.49**	**0.090**	**0.081**	**0.009**	
"	13	1.31	0.099	0.083	0.016	
"	14	1.52	0.107	0.086	0.021	
"	15	1.49	0.107	0.092	0.015	

When the phenyluraminocystine was introduced parenterally, however (Tables III and IV), no oxidation of the sulfur of the molecule occurred. All of the "extra" sulfur eliminated appeared in the unoxidized sulfur fraction and no increase in sulfate sulfur was evident. The total amount of "extra" sulfur recovered was, however, somewhat less than in the experiments in which the phenyluraminocystine was fed. Cystine when injected in amounts comparable to those of the phenyluraminocystine used did not give rise to any appreciable increase in the unoxidized sulfur fraction. The animal body was able to oxidize completely the sulfur of injected cystine.

In one experiment (Table V) the phenyluraminocystine was fed as a suspension in milk. In this case also no oxidation of the compound took place, and all of the "extra" sulfur was eliminated as unoxidized sulfur. As was to be expected the rate of elimination was slower than in those experiments in which the phenyl-

TABLE IV.

Rabbit D. Female, grey. Weight 1.98 kilos. Daily diet: 150 cc. of milk and 30 gm. of oats.

Date.	Total S.	Total SO_4 S.	Unoxidized S.	Remarks.
1920	gm.	gm.	gm.	
Dec. 13	0.075	0.057	0.018	
" 14	0.072	0.059	0.013	
" 15	0.065	0.052	0.011	
" 16	**0.109**	**0.096**	**0.015**	0.501 gm. cystine as hydrochloride subcutaneously (S = 0.134 gm.).
" 17	0.082	0.067	0.015	
" 18	0.083	0.067	0.016	
" 19	**0.150**	**0.058**	**0.092**	1.0 gm. phenyluraminocystine subcutaneously as sodium salt (S = 0.134 gm.).
" 20	0.075	0.053	0.022	

TABLE V.

Rabbit E. Female, grey. Weight 1.68 kilos. Daily diet: 150 cc. of milk, 10 gm. of sugar, and 30 gm. of oats.

Date.	Total S.	Total SO_4 S.	Unoxidized S.	Remarks.
1921	gm.	gm.	gm.	
Jan. 22	0.037	0.027	0.010	
" 23	0.037	0.026	0.011	
" 24	0.033	0.025	0.008	
" 25	0.043	0.032	0.011	
" 26	**0.102**	**0.029**	**0.073**	1.0 gm. phenyluraminocystine suspended in milk.
" 27	0.067	0.031	0.036	
" 28	0.043	0.029	0.014	
" 29	0.038	0.026	0.012	

uraminocystine was fed as the soluble sodium salt. More "extra" sulfur appeared in the urine on the day following the administration than in the preceding experiments.

The results appear to demonstrate conclusively that, when deamination of the cystine molecule was prevented, the oxidation of the sulfur of the molecule did not take place normally. It

seems probable that in the case of cystine as with the aromatic amino-acids, complete oxidation of the molecule is connected with the deamination process or the further oxidation of the products of deamination. The reason for the slight degree of oxidation when the sodium salt of phenyluraminocystine is administered *per os* is not evident, but we believe this to be the result directly or indirectly of some bacterial action. The study of some other derivatives of cystine in which the amino group is "protected" is in progress.

SUMMARY.

The sulfur of phenyluraminocystine when administered subcutaneously as the sodium salt was not oxidized in the organism of the rabbit, but was eliminated as "extra" unoxidized sulfur. Cystine under the same experimental conditions did not increase the unoxidized sulfur content of the urine. When the sodium salt of phenyluraminocystine was fed to rabbits, a limited oxidation of the sulfur fraction of the molecule, resulting in a slight increase in the elimination of sulfate sulfur occurred, although the greater part of the sulfur administered was recovered in the unoxidized sulfur fraction. Since uramino-acids are not broken down in the organism, these results are believed to indicate that the oxidation of the sulfur of the cystine molecule is connected with the process of deamination or the oxidation of the deamination products.

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THE VITAMINE CONTENT OF MICROORGANISMS IN RELATION TO THE COMPOSITION OF THE CULTURE MEDIUM.

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The Vitamine Content of Yeast.

That yeast is a valuable source of the antineuritic factor was first demonstrated by Schaumann. It has also been claimed that the growth-promoting, water-soluble B substance is abundant in yeast, and it is believed by some workers that these factors are identical, a hypothesis to which we shall return later. Moreover, yeast and yeast extracts have already been introduced into therapeutics, and the latter are also used in food (soup cubes and so forth).

The following observation led us to examine the vitamine content of yeast in regard to the composition of the culture medium.

In my attempts to isolate the antineuritic factor from an aqueous solution of extract of rice polishings, among other methods which I tried, I removed the sugars through the agency of yeast. But the unwished for result was that the medium had lost its antineuritic properties. This experience, in combination with the fact that bakers' as well as brewers' yeast is obtained by cultivating them in media originally containing vitamine, gave rise to the supposition that the yeast cell may not be able to synthesize the vitamine but may take it as such from the medium.

First, we cultivated *Saccharomyces*, isolated from bakers' yeast in vitamine-free media, namely in glucose-peptone broth as well as in a synthetic medium, containing only well known chemical compounds. For this purpose we prepared a solution of

0.5 gm. of NaCl, 0.2 gm. of KH_2PO_4, 0.05 gm. of $CaSO_4$, 0.02 gm. of $MgSO_4$, 5 mg. of $FeSO_4$, 1 mg. of $MnCl_2$, 1 mg. of ZnCl, 0.3 gm. of NH_4Cl, and 5 gm. of dextrose per 100 gm. of water. Henceforth we shall indicate this solution as "synthetic wort." It proved to be suitable for the growth of yeast.

The yeast species which we cultivated at 27°C. in vitamine-free media, proved in experiments on polyneuritic fowls to fail in curative effect. In this connection an old experiment of one of us may be called to mind, according to which polished rice, after its preparation, in the cooked state, into a sweet meat ("tapej") by the addition of Chinese rice yeast ("ragi"), nevertheless remains deficient in the antineuritic factor.

On the other hand, control experiments with the same species of bakers' yeast, cultivated at 27°C. in aqueous solution of extract of rice polishings (sp. gr. 1.045), after washing with physiological salt solution in order to remove the adherent traces of the medium, gave a distinctly positive result. This aqueous extract had been previously divided into two portions, one of which was boiled for a short time only and then filtered and inoculated with the yeast, whereas the other portion was heated before filtering for 1 hour in the autoclave at 120°C. in order to destroy the antineuritic factor. Both of these yielded highly active yeast, but the liquids, separated from the yeast at the end of the fermentation, were found to be inactive.

The same experiments were repeated with other materials, but the result was the same as before. This time we chose a *Saccharomyces* originating from beer yeast, and for media, beer-wort and also a "synthetic wort" so far different from the former that ash of beer-wort, in the same concentration as in beer-wort took the place of the artificial mineral salt mixture, while the reaction of the medium was made slightly acid by the addition of lactic acid.

We took the beer-wort from the brewery in two respective stages of its preparation. The sample of the first stage was heated for a short time only at about 75°C., and proved in experiments on fowls to contain the antineuritic factor. The second sample was boiled for about 2 hours and was hopped. This sample was found to be practically devoid of antineuritic properties. The pure culture from beer yeast which we used in these

experiments, was a so called bottom yeast and the fermentation of the three liquids took place at 6-7°C.

As has already been indicated, the "synthetic wort" yielded yeast without any marked curative power against polyneuritis of fowls whereas the two samples of beer-wort produced a highly, active amount of yeast, the liquids themselves on the contrary proving to be inactive at the end of the fermentation.

It seems, therefore, that yeast not only takes eventually its antineuritic factor as such from the culture medium but that it is not even capable of synthesizing the vitamine unless the medium contains at least the products of decomposition of the vitamine by heating.

In the light of the above mentioned hypothesis this conclusion does not agree with that of Nelson, Fulmer, and Cessna[1] who in experiments on young rats found that yeast can synthesize the growth-promoting, water-soluble B substance in a medium consisting merely of aqueous solution of mineral salts, NH_4Cl, and cane-sugar. These apparently contradictory facts give us reason to doubt whether the antineuritic factor and the water-soluble B substance are really identical. As neither of them has thus far been isolated in an unquestionably pure state, their suggested identity is principally based on the presence or absence of both in the same foodstuffs as also on their conduct towards the same physical and chemical agents. Mitchell,[2] however, drawing attention to the fact that the correlation in all these respects is far from being without exceptions, concludes that in evaluating the data on the occurrence and properties of the two vitamines, there seems to be very good reason for doubting their identity.

Since our experimental results made it probable that the yeast cell may take its antineuritic factor as such from that of the culture medium, although the conclusive proof that the antineuritic factor and water-soluble B are indeed the same has not yet been furnished, the question arose whether from its minute dimensions the yeast cell was able to absorb that factor in a similar way as was already known with regard to charcoal and fullers' earth. In order to decide this question we took advantage of our experience

[1] Nelson, V. E., Fulmer, E. I., and Cessna, R., *J. Biol. Chem.*, 1921, xlvi, 77.

[2] Mitchell, H. H., *J. Biol. Chem.*, 1919, xl, 399.

that yeast, when cultivated in a "synthetic wort," is devoid of the antineuritic factor. Therefore, such yeast was put into vitamine-containing beer-wort, and the mixture shaken for about half an hour at a low temperature. During this time there occurred no noticeable growth of yeast, as could be controlled by sedimenting tests with the hematocrit. After shaking, the yeast was separated from the medium by centrifuging and washing. In experiments on fowls, it did not show any curative effect.

The conclusion may be drawn that the process by which the yeast cell takes its vitamine from the medium is not properly of a physicochemical nature; *i.e.*, an *adsorption*, but a relatively slow, biological one—a *resorption*.

The Vitamine Content of Bacillus coli communis.

According to some authors,[3] various bacterial cultures contain water-soluble B. We did not succeed in confirming these experiments in regard to the antineuritic factor.

A *coli* stock, isolated from the intestines of a fowl, was cultivated at an adequate temperature (42°C.) in a highly active aqueous extract of rice polishings, which had been cautiously sterilized by discontinuous heating at 75°C. After 3 days cultivation the amount of *coli* was collected on a bacterial filter and cleansed by washing. Unlike yeast gained under similar conditions it showed no marked antineuritic properties.

SUMMARY.

Though with some reserve, arising from our insufficient knowledge concerning the composition of the antineuritic factor, we may conclude as follows:

1. The yeast cell can take its antineuritic factor as such from the culture medium. This is not merely an absorption process.

2. The yeast cell is not able to synthesize, in the strict sense of the word, the antineuritic factor, but only to regenerate it after it has been denatured by heating.

3. *Bacillus coli communis*, even after having been cultivated in a medium which contains the antineuritic factor, remains devoid of this vitamine.

4. The antineuritic factor and the growth-promoting, water-soluble B substance are not identical.

[3] Pacini, A. J. P., and Russell, D. W., *J. Biol. Chem.*, 1918, xxxiv, 43. Bierry, H., *Compt. rend. Soc. biol.*, 1919, lxxxii, 307.

A STUDY OF THE EFFECT PRODUCED ON THE COMPOSITION OF MILK BY THE ADMINISTRATION OF CERTAIN INORGANIC AND ORGANIC SUBSTANCES.

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Attempts to modify the composition of milk by experimental means have been numerous and as a rule unsuccessful. To reopen the subject would therefore scarcely appear justifiable except for the fact, that in virtue of the developments in microchemical blood analysis which have taken place during the last decade, we felt ourselves able to approach the problem from a new standpoint. Observations regarding the passage into the milk of drugs administered by mouth have been made for many years, and while such observations are mainly of a qualitative nature, and give as a rule no idea of the amount of absorption, they still indicate the possibility of producing experimentally changes in the mammary secretion by the production of changes in the composition of the blood.

It is now generally conceded that it is possible by dietary measures to influence the concentration of certain of the inorganic and organic non-protein constituents of the blood, whereas in the case of certain other normally occurring constituents forced feeding either produces no demonstrable effect or at most a transitory rise. In the first class urea and phosphates may be mentioned, whereas in the latter striking examples are to be found in sodium chloride and in the salts of calcium.

In view of these facts it would seem that it should in most cases be possible to predict the result of feeding experiments made with the object of producing changes in the composition of the milk (at least as regards a single constituent), provided data are on hand

concerning the possibility of altering the concentration of this specific constituent in the blood.

The experiments described below were undertaken with the purpose of obtaining experimental proof of the validity of the above hypothesis. In this paper are given the results obtained by the administration of urea and calcium chloride.

Our experimental methods were as follows:

An appropriate amount of the substance whose absorption was to be studied was dissolved in about 300 cc. of water and poured down the animal's throat without the use of stomach tube. All samples of blood were taken by venepuncture from the external jugular vein.

The analytical methods used were as follows:

For Milk.

Urea.—By the urease method according to the technique described by Denis and Minot (1).

Calcium.—By Lyman's (2) method, slightly modified, as an extended experience has shown us that in experimental work better results are obtained if several standards of varying strength are provided instead of the single standard recommended by Lyman.

For Blood.

Urea.—By the method of Folin and Wu (3).

Calcium.—By the method of Lyman (2).

Absorption of Urea.

The ability of the body rapidly to absorb ingested urea is now too well known to require comment. It has been shown by Marshall and Davis (4) that when urea is injected intravenously it is stored in the muscles and in the various organs of the body in amounts approximately equal to the concentration found in the blood. Of recent years much experimental work has been carried out on the effects of high and low protein diets on the concentration of blood urea; in general the results of this work lead us to believe that it is possible, even in subjects with normal kidney function to increase the concentration of blood urea by relatively short

periods of high protein feeding, and to decrease it by the use of low nitrogen diets. It was also shown some years ago by Denis and Minot (5) that by the administration of diets high in protein it was possible to obtain from cows, milk of high urea content, whereas the same animals when fed on low protein diets produced milk containing relatively small amounts of urea. These studies were unfortunately not accompanied by observations of blood urea. In Experiments 1, 2, and 3, the results of which are tabulated in Tables I, II, and III we have studied the relative effects of large doses of urea on the blood and milk.

TABLE I.

Absorption of Urea.

Experiment 1.—Goat 1, weight 31.8 kilos. An old animal, whose average yield of milk for 24 hours was about 150 cc.

Time.	Urea administered.	Volume of milk.	Urea N per 100 cc. of milk.	Increase of urea N over normal.
1920	*gm.*	*cc.*	*mg.*	*per cent*
May 24, 8.15 a.m.		65	17.8	
9.00 "	5			
10.00 "	5			
11.00 "	5	15	19	6.9
12.00 m.	5			
1.00 p.m.		7	26	46.0
3.00 "		3	95	433.7
May 25, 9.00 a.m.		37	29	62.9

As will be seen such treatment causes a rapid rise in the urea nitrogen fraction of both blood and milk. If we disregard the results of Experiment 1 in which no blood examinations were made, it will be noted that the urea content of the milk had increased to more than 30 per cent of its former value in 1 hour in Experiment 2, whereas in Experiment 3 a similar result was obtained in these specimens of milk taken 3 hours after the administration of urea. A similar relation exists between the two experiments as regards the final concentration of urea attained. In Experiment 2 the maximum figures were obtained 6 hours and 45 minutes after the administration of the initial dose, the rise being represented by an increase of 163 per cent in the milk and 206 per cent in the

plasma, whereas in Experiment 3 after 11 hours the rise in the urea nitrogen of the milk was represented by an increase of 97 per cent.

TABLE II.

Absorption of Urea.

Experiment 2.—Goat 2, weight 22.5 kilos. A young animal in the first month of lactation. The average yield of milk for 24 hours was about 1 liter.

Time.	Urea administered.	Volume of milk.	Urea N per 100 cc.		Increase of urea N over normal.	
			Milk.	Plasma.	Milk.	Plasma.
1920	*gm.*	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*
June 9, 9.00 a.m....		600	14.4	15.0		
9.15 " ...	5					
10.15 " ...	5					
11.15 " ...	5	96	19		31.9	
12.15 p.m....	5					
1.30 " ...		40	37		15.69	126.0
3.15 " ...		40	38		163.8	206.6
10.00 " ...		170	25.5		77.0	
June 10, 8.30 a.m....		420	12.4			

TABLE III.

Absorption of Urea.

Experiment 3.—Goat 3, weight 36 kilos. An old animal in the first month of lactation. The average 24 hour yield of milk was about 700 cc.

Time.	Urea administered.	Volume of milk.	Urea N per 100 cc.		Increase of urea N over normal.	
			Milk.	Plasma.	Milk.	Plasma.
1920	*gm.*	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*
June 22, 6.00 a.m....		350	13.4	20		
6.10 " ...	5					
7.00 " ...	5					
8.10 " ...	5					
9.10 " ...	5	65	17.6		31.3	
12.00 m......		30	19	30	44.7	50.0
3.00 p.m....		44	19	31	41.7	55.0
5.00 " ...		50	26.4		97.0	
June 23, 9.00 a.m....		320	29		116.4	
5.00 p.m....		200	15.3		14.1	
June 24, 9.00 a.m....		310	17		27.5	

Absorption of Calcium Chloride.

Feeding experiments made with the object of modifying the calcium content of milk have usually been unsuccessful (6). Similar negative results have been the reward of investigators who have attempted to increase the calcium content of the blood by the administration of calcium salts (7). It is not surprising, therefore, that our attempts to change the calcium concentration of the milk by feeding large doses of calcium chloride (as described in the tabulation of the results obtained in Experiments 7, 8, and 9, Tables IV, V, and VI) should also have yielded negative results as regards both milk and blood.

TABLE IV.

Absorption of Calcium Chloride.

Experiment 7.—Goat 1.

Time.	$CaCl_2$ administered.	Volume of milk.	Per 100 cc. of milk.		Increase.	
			Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*
May 29, 9.00 a.m....		85	15.9	156		
9.30 " ...	2					
10.30 " ...	2		158	156	0	0
11.30 " ...	2					
12.30 p.m....	2	37	158	180	0	15.3
3.30 " ...		13	158	180	0	15.3

In Experiments 7 and 8 unmistakable increase in the chloride concentration in the plasma and milk was observed. In Experiment 9, however, this increase was confined to the plasma, no change being noted in the milk.

As it occurred to us that our negative results in the case of these experiments in which calcium chloride was administered by mouth might be due to slow absorption of this salt from the stomach and small intestine we performed a final experiment in which 1.87 gm. of calcium chloride contained in a volume of 75 cc. were injected intravenously. The duration of the injection was 3 minutes. The plasma from a sample of blood taken 17 minutes after the end of the injection showed an increase in the calcium content of 150 per cent, 3 hours later the plasma calcium was still 48 per cent above the initial figure. Even with this unmistakable

increase in the calcium concentration of the blood, however, no increase was noted in the calcium concentration of several samples of milk removed at intervals during the day.

TABLE V.

Absorption of Calcium Chloride.

Experiment 8.—Goat 2.

Time.	$CaCl_2$ administered.	Volume of milk.	Per 100 cc.				Increase.			
			Milk.		Blood.		Milk.		Blood.	
			Ca	Cl	Ca	Cl	Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
June 29, 8.50 a.m.		320	160	140	8.6	354				
9.00 "	4									
10.00 "	4									
11.00 "	4									
12.00 m.	4	44	161	164	8.5	360	0	17.1	0	1.6
3.00 p.m.		43	160	169	8.6	380	0	20.7	0	10.1
June 30, 9.00 a.m.			160			361	0			1.6

TABLE VI.

Absorption of Calcium Chloride.

Experiment 9.—Goat 3.

Time.	$CaCl_2$ administered.	Volume of milk.	Per 100 cc.				Increase.			
			Milk.		Plasma.		Milk.		Plasma.	
			Ca	Cl	Ca	Cl	Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
June 29, 8.40 a.m.		420	163	169	8.7	360				
9.00 "	4									
10.00 "	4									
11.00 "	4									
12.00 m.	4	72		169	8.6	402		0	0	10.1
3.10 p.m.		60	164	180	8.7	432	0	0	0	20.0
June 30, 9.00 a.m.		380	164	170	8.6	360	0	0	0	0

The results of the experiments described above would appear to lend support to the hypothesis outlined in the opening paragraphs of this paper. If we believe that the mammary tissue may act

as a temporary storage place for certain substances that are not rapidly excreted our work would suggest that both the chlorine ion and urea may be found in increased amounts in the milk when their concentration in the plasma, and probably also in the mammary tissue, rises to a high level. The calcium ion on the other hand apparently acts in an entirely different manner. The cause of this difference in behavior may be ascribed to the relative toxicity of calcium with the resultant lowering of the dosage, or to the fact that calcium cannot be retained in the mammary tissue but is

TABLE VII.

Absorption of Calcium Chloride.

Experiment 10.—Goat 3.

Time.	$CaCl_2$ injected.*	Volume of milk.	Per 100 cc.				Increase.			
			Milk.		Plasma.		Milk.		Plasma.	
			Ca	Cl	Ca	Cl	Ca	Cl	Ca	Cl
1920	*gm.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
July 7, 9.30 a.m.		360	200	164	128	350				
10.35 "	Injected 1.87 gm. $CaCl_2$.									
10.55 "					32	400			150	14
11.55 "		55	200	175			0	6.6		
1.50 p.m.		35	196	180	19	350	0	9.7	48	0
4.10 "		30	200	173	13	350	0	6.4	1.4	0
July 8, 9.10 a.m.		200	201	180			0	9.7		

* At 10.30 injected into the external jugular vein 75 cc. of a 25 per cent solution of calcium chloride.

excreted almost immediately by the intestine and kidney. In view of the unmistakable increase in the chlorine concentration of the milk of the animals receiving calcium chloride we feel inclined to accept this latter view.

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THE THERMOSTABLE ACTIVE AGENT OF PIG'S PANCREAS.

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More than a year ago the writer stated that yeast nucleic acid is decomposed into its nucleotides by a boiled aqueous extract of pig's pancreas without giving rise to any increase in the acidity of the solution; and the crucial bearing of this circumstance upon the chemical constitution of yeast nucleic acid was discussed in detail.[1]

The context of the article and of other writings on the subject indicated clearly that the word "acidity" was intended to mean "titratable acidity" (not necessarily hydrogen ion concentration)[2] and the implication was definite that titrations had been made. In the experimental part (which was not otherwise complete) no specific description was given of a titration, the principal description being of an experiment dealing only with hydrogen ion concentration. This experiment Levene[3] now criticizes in the following words:

"The second experimental proof of Jones' theory is the following: By a pancreas enzyme, yeast nucleic acid was cleaved to its nucleotides. At the starting point of the experiment the hydrogen ion concentration of the reacting mixture was brought to pH = 6.4, and at the end of the experiment there was no apparent change of the color of the indicator added to

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[1] Jones, W., *Am. J. Physiol.*, 1920, lii, 203.

[2] See Jones, W., Monograph on biochemistry, London, 1920, 47, "the increased acidity caused by the decomposition of two grams of nucleic acid should require about 8 cc. of tenth normal sodium hydroxide for neutralization toward phenolphthalein. There is no doubt about this. The nucleotides imitate the conduct of phosphoric acid toward alkalis and indicators."

[3] Levene, P. A., *J. Biol. Chem.*, 1921, xlviii, 119.

the original solution. Hence the author concludes that no acid radicles could be liberated as the result of the hydrolysis. The reasoning is not correct. According to either theory, nucleic acid is a polyphosphoric acid and when brought to a pH = 6.4, it possesses considerable buffer effect. Furthermore, each nucleotide is a comparatively weak acid and when liberated does not affect the hydrogen ion concentration of the buffer very markedly. Since the dissociation constant of the nucleotides has not been measured, it is not possible to express the reaction in quantitative terms. Experimentally, however, we convinced ourselves that when a solution of guanosinphosphoric acid is brought to a pH = 6.4, it stands the addition of an equal volume of a solution of free guanosinphosphoric acid of the same concentration before any change of color of the indicator can be noticed. Taking further into consideration the fact that a solution of nucleic acid is not perfectly colorless, that an extract of the pancreas always contains a considerable quantity of phosphates and also is not colorless, one easily realizes that the argument of Jones carries but little weight."

This criticism would be more plausible if the following were ignored:

1. The pale yellow color of a boiled pancreas extract matches well the alkaline color of the indicator used (brom-cresol). The change of this indicator from its purple color to yellow can be detected just as well in a pale yellow solution as in distilled water when a proper arrangement for the observation is made.

2. A 2 per cent solution of Merck's yeast nucleic acid is scarcely colored. But aside from this, the specimen of yeast nucleic acid that we have been using in this laboratory is snow-white and forms a colorless 2 per cent solution. A method will be described later for its preparation from yeast and from the various commercial sources of yeast nucleic acid.

3. All four of the nucleotides are strong enough acids to turn methyl orange.

4. Levene's experiment with guanosinphosphoric acid has been repeated with both adenine nucleotide and guanine nucleotide. The results obtained are strikingly different from the result given by Levene.[4] 15 cc. of a 7 per cent solution of adenine nucleotide in hot water were cooled, colored with brom-cresol, and brought to a hydrogen ion concentration of pH = 6.4. The purple solution was divided into two equal parts, one of which

[4] Levene does not state what indicator he used. This is of vital importance.

was used for comparison while the other was carefully treated with a 7 per cent solution of adenine nucleotide. The first drop produced a color change that could doubtfully be detected; the second drop produced an unmistakable change in the color of the indicator; the third drop so changed the indicator that its predominant color was yellow. Essentially the same results were obtained with guanine nucleotide.

5. No *titratable acidity* is produced when yeast nucleic acid is decomposed into its nucleotides by the action of boiled extract of pancreas, as the following experiments will show.

A boiled aqueous extract of pig's pancreas was prepared according to the directions previously given[1] and 3 gm. of yeast nucleic acid were dissolved in 150 cc. of the warmed extract.[5] This solution, in which the presence of the nucleic acid could be shown even after excessive dilution, was quickly cooled to the room temperature, placed in a burette, and compared with a tenth normal solution of sodium hydroxide using phenolphthalein as an indicator with the usual precautions. The results of ten titrations given in Table I show that for 5 cc. of the nucleic acid solution close to 5.60 cc. of the alkali were required for neutralization toward phenolphthalein.

The remainder of this nucleic acid solution was preserved with a few drops of chloroform and allowed to digest in the thermostat at 38° for 24 hours. At the end of this time the nucleic acid had entirely disappeared, having been converted into its nucleotides. After cooling to the room temperature the solution was placed in a burette and again compared with the tenth normal solution of sodium hydroxide, using phenolphthalein as an indicator with the usual precautions. The results of eight titrations given in Table I show that for 5 cc. of the digested solution close to 5.75 cc. of the alkali were required for neutralization toward phenolphthalein.

Weighed portions of adenine nucleotide were then added to 5 cc. portions of the digested fluid and the product was titrated as before. The titratable acidity was found increased by the theoretical demand.[6]

[5] The nucleic acid dissolves readily in the warmed extract without the addition of alkali.

[6] The experiment here reported was done after Levene's article appeared, but the results do not materially differ from those of numerous older and recent experiments.

The two formulas which have been proposed in turn by Levene for yeast nucleic acid and the one proposed by Thannhauser and Sachs[7] demand the production of titratable acidity in each of the ten experiments corresponding to 4.60, 2.31, and 5.25 cc. of tenth normal sodium hydroxide, respectively. But no titratable acidity at all was found, although adenine nucleotide exhibits its theoretical titratable acidity in a boiled aqueous extract of pig's pancreas.

It is true that no experimental evidence exists to show the points at which the individual nucleotide groups of yeast nucleic

TABLE I.

Before digestion (nucleic acid).			After digestion (nucleotides formed).			
Extract + nucleic acid used.	Alkali required.	Alkali calculated for 5 cc. of extract.	Extract + nucleotides formed.	Adenine nucleotide added.	Alkali required.	Alkali calculated for 5 cc. of extract.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*cc.*	*cc.*
3.42	3.80	5.55	3.17		3.55	5.60
3.81	4.27	5.60	3.81		4.19	5.50
3.60	4.07	5.65	3.30		3.63	5.50
3.12	3.43	5.50	4.00		4.56	5.70
4.18	4.68	5.60	4.12		4.70	5.70
3.97	4.49	5.65	3.30		3.70	5.60
3.51	3.90	5.55	3.21		3.53	5.50
3.70	4.07	5.50	3.60		3.96	5.50
4.07	4.60	5.65	3.19	50	6.25	
4.13	4.71	5.70	3.34	50	6.42	
4.07	Used for comparison.		3.18	Used for comparison.		
Mean		5.60	Mean			5.75

acid are joined to one another; but there exists abundant experimental evidence to show the points where they are *not* joined. The nucleotide linkages do not involve any one of the phosphoric acid groups and no formula for yeast nucleic acid can be accepted in which this kind of nucleotide linkage is assumed. The carbohydrate formula that I have used is arrived at only by exclusion and is intended specially to indicate the points where the nucleotide linkages *do not exist.*

[7] Thannhauser, S. J., and Sachs, P., *Z. Physiol. Chem.*, 1921, cxii, 189.

In the same article Levene[3] criticizes another kind of experimental evidence that I have adduced, and in the following words:

"The curve expressing the rate of hydrolysis of yeast nucleic acid is identical with that of a mixture of the four nucleotides. Accepting the experiment as correct, what does it demonstrate? It proves that the union between individual nucleotides is more labile than that between the phosphoric acid and the carbohydrate in each nucleotide. It is then self-evident that the first step in the hydrolysis of the nucleic acid molecule is the formation of four nucleotides. The further progress of hydrolysis of the nucleic acid is the same as of four nucleotides."

I raised this question myself long ago and dealt with it in the following words.[8]

"It is of course possible to draw other conclusions that can be adjusted to the facts, but they all involve the assumption of curious coincidences and compensations which would cause phosphoric acid to be liberated with equal ease from different kinds of linkage, or that the liberation of phosphoric acid from one kind of linkage is excessively slower than from another. After careful consideration of such matters, we believe we have drawn the correct conclusion."

The contents of the present article show that this statement did not exhibit poor critical judgment.

In Levene's article[3] is contained the following unfortunate sentence.

"It is peculiar that Jones, in the latest edition of his monograph, in discussing the theories of the constitution of yeast nucleic acid, does not at all refer to the theory of the present writer."

It is not peculiar. I knew at the time what is contained in the present article. Had I known what is contained in Levene's recent communication,[3] I should not have referred to his theory of the constitution of *thymus* nucleic acid.

CONCLUSION.

The existence in the pancreas extract of an easily detected thermostable agent which decomposes yeast nucleic acid only as far as its nucleotides, is more interesting in its physiological significance than for any light that it throws upon the chemical

[8] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 126.

constitution of nucleic acid. The first alteration that yeast nucleic acid undergoes in its decomposition by tissue extracts is assumed to be the production of nucleotides. How then does it happen that extract of pig's spleen and of other tissues, that do not contain the thermostable agent in question, can nevertheless bring about the progressive decomposition of nucleic acid with the formation of free phosphoric acid and free purine bases?

It would appear either that there are two ferments which can decompose nucleic acid into its nucleotides, one of which is destroyed by heat, or that the decomposition of nucleic acid by tissue extracts does not proceed along conventional lines. Examination of the matter is now proceeding.

A RAPID COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF THE INORGANIC PHOSPHORUS IN SMALL AMOUNTS OF SERUM.

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Principle of the Method.

The principle of the method consists in the precipitation of the phosphorus, in a trichloroacetic acid extract of serum, as strychnine phosphomolybdate, the isolation of the precipitate by the use of a centrifuge and small quantities of water, and the subsequent development of a brilliant green color produced by the reduction of the molybdenum present in the precipitate. The reduction is accomplished by the use of potassium ferrocyanide and HCl.

The Method.

Precipitation of Protein.—1 cc. of serum is transferred to a 15 cc. centrifuge tube and to this are added 5 cc. of a 6 per cent solution of trichloroacetic acid. The mixture is thoroughly mixed with the aid of a glass rod and allowed to stand for 4 minutes. It is then centrifuged for 4 to 5 minutes at about 1,500 revolutions per minute and the supernatant fluid poured off.

Precipitation of Phosphorus with the Strychnine Molybdate Reagent.—5 cc. of the supernatant fluid are measured into an ordinary 15 cc. graduated centrifuge tube, the outside diameter of which is 6 to 7 mm. at the 0.1 cc. mark. Water is added to bring the volume to 6 cc., followed by 2 cc. of the strychnine molybdate reagent which should be added drop by drop, and the tube shaken three or four times during the procedure. The contents of the tube are then thoroughly mixed by holding the tube at the upper

end and tapping the lower end with the finger giving it a circular motion. The contents are allowed to stand for 10 minutes during which time they are thoroughly mixed twice as outlined above.

Washing of Precipitate.—After the 10 minutes has elapsed the tube is centrifuged at 1,500 revolutions per minute for 3 minutes, the supernatant fluid is poured off and the mouth of the tube wiped with a dry cloth. 3 cc. of water are allowed to run down the sides of the tube which removes any adherent supernatant fluid. The residual supernatant fluid (about 0.1 cc.) is thoroughly mixed with the added water by tapping the lower end of the tube with the finger giving it a circular motion, while the precipitate is disturbed as little as possible. The mixture is centrifuged for 1 minute at 1,500 revolutions per minute, the supernatant fluid is poured off and the above procedure repeated, making two washings in all.

Development of Color.—After the final supernatant fluid has been removed, 2 cc. of a 1 per cent solution of NaOH are added and the contents mixed with the aid of a glass rod. This causes all the precipitate to go into solution. Water is added to 10 cc. and the contents are transferred to a 100 cc. glass-stoppered volumetric flask. Traces of the solution remaining in the centrifuge tube are washed into the flask by means of two lots of 10 cc. of water, so that the total volume of fluid in the flask is 30 cc. 20 cc. of a 20 per cent solution of potassium ferrocyanide are then added, followed by 10 cc. of concentrated HCl. The flask is inverted two or three times and allowed to stand 10 minutes. Water is added to 100 cc., the contents are thoroughly mixed, and the color is read in the colorimeter against the standard.

Preparation of the Standard.—1 cc. of a solution of KH_2PO_4 containing 5 mg. of P per 100 cc. (219.3 mg. of KH_2PO_4 (Merck) in 1,000 cc.) is measured into a graduated centrifuge tube, which contains 5 cc. of water, and the contents are thoroughly mixed. 2 cc. of the strychnine molybdate reagent are then added drop by drop. This step, and the washing of the precipitate and the development of the color, are carried out at the same time and in the same manner with both the standard and the unknown.

The Volume of the Precipitate.—The amount of precipitate obtained in the standard solution after it is centrifuged is almost exactly 0.1 cc. of volume. If the amount of precipitate obtained

in the unknown is 0.2 cc. or more, its solution (in 1 per cent NaOH) should be made up to a definite volume in the centrifuge tube and an aliquot taken which would contain approximately 0.1 cc. of the precipitate. If the amount of precipitate obtained in the unknown is about one-half the amount in the standard, its solution should be made up to 5 cc. and transferred to a 50 cc. volumetric flask with the use of two lots of 5 cc. of water. In all the subsequent steps the volumes used should be halved.

Calculation.—When the unknown is made up to 100 cc. and the standard solution is set at 20 in the colorimeter the calculation is as follows:

$$\frac{20}{\text{Unknown}} \times 6 = \text{mg. of P per 100 cc. of serum.}$$

When the unknown is made up to 50 cc. the result is divided by 2.

Preparation of the Strychnine Molybdate Reagent.

Solution A is prepared by dissolving 50 gm. of ammonium molybdate in 150 cc. of warm water. If not clear this solution should be filtered.

Solution B consists of 2 volumes of concentrated HNO_3 and 1 volume of water.

Solution C is prepared by pouring 1 volume of Solution A into 3 volumes of Solution B.

Solution D consists of strychnine nitrate 7.5 gm., water to 500 cc. The water may be warmed to facilitate solution.

1 volume of Solution D is poured into 3 volumes of Solution C. This constitutes the strychnine molybdate reagent. The reagent should stand 24 hours before it is used. It will keep for at least 1 month. After the reagent has stood for 1 or 2 days a slight precipitate forms and when this occurs it should be filtered. 2 cc. of the reagent will precipitate 0.2 mg. of P.

Protocols.

The development of the color on the addition of potassium ferrocyanide and HCl is in direct proportion to the amount of molybdenum present, as long as that amount does not exceed double the quantity, or is not less than half the quantity present

in the standard. A solution of strychnine phosphomolybdate was prepared so that 2 cc. contained approximately the amount of precipitate obtained from 0.05 mg. of P (1 cc. of the standard solution). 1, 2, and 4 cc. of this solution were placed in 100 cc. volumetric flasks, the volumes made up to 30 cc. with water, and potassium ferrocyanide and HCl added. After 10 minutes the flasks were made up to volume and the color was read in the colorimeter with the flask containing 2 cc. of the solution taken as the standard at 20. Reading obtained with 1 cc. of solution

TABLE I.

Estimation of Known Amounts of Phosphorus in a Solution of KH_2PO_4.

Amount of solution.	Quantity of P.	Volume of precipitate after centrifuging.	Size of flask.	Amount of potassium ferrocyanide added.	Amount of HCl added.	Reading.	Theory.	Error.
cc.	*mg.*	*cc.*	*cc.*	*cc.*	*cc.*			*per cent*
0.25	0.0125	Less than 0.05.	50	10	5	40.8	40.0	−2
0.5	0.025	About 0.05.	50	10	5	20.4	20.0	−2
1.0	0.05	0.1	100	20	10	Standard = 20		
2.0	0.10	0.2	200	40	20	19.8	20.0	+1
3.0*	0.15	0.3	100	20	10	19.6	20.0	+2
4.0*	0.20	0.4	100	20	10	19.8	20.0	+1

* The solutions of the precipitate obtained from the 3 and 4 cc. samples were made up to 6 and 8 cc., respectively, and 2 cc. aliquots transferred to the 100 cc. flasks.

= 39. Theory 40. Error + 2.5 per cent. Reading obtained with 4 cc. of solution = 10.3. Theory 10.0. Error −3 per cent.

The amount of precipitate obtained and the development of the color are in direct proportion to the amount of P present in the sample in quantities varying from 0.0125 to 0.2 mg. of P. This is shown in Table I.

Trichloroacetic acid in the concentrations used does not interfere with the precipitation and determination of the phosphorus present.

The presence of Na, K, Ca, and Mg in concentrations comparable to those found in serum and also the following organic com-

pounds do not interfere with the precipitation and determination of the phosphorus: Dextrose 300 mg. per 100 cc., urea 300 mg.

TABLE II.

Recovery of Phosphorus Added to Serum.

Serum.	Inorganic P present.	P added.	Total inorganic P found.	Total inorganic P present.	Error.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
10	0.078	0.0125	0.088	0.090	−2.2
10	0.078	0.025	0.102	0.103	−1.0
10	0.078	0.050	0.127	0.128	−0.8
21	0.050	0.0125	0.062	0.062	±0.0
21	0.050	0.025	0.073	0.075	−2.6
21	0.050	0.050	0.105	0.100	+5.0

TABLE III.

Inorganic Phosphorus Content of Normal Adult Serum (Twenty-Two Consecutive Determinations).

Serum.	Inorganic P per 100 cc.	Serum.	Inorganic P per 100 cc.
	mg.		*mg.*
30	3.7	41	3.2
31	4.0	42	3.9
32	3.7	43	3.9
33	3.5	44	3.7
34	3.7	45	3.8
35	3.7	46	3.7
36	3.6	47	4.3
37	3.5	48	4.0
38	4.2	49	3.6
39	3.7	50	3.7
40	4.0	51	3.8
		52	3.7

per 100 cc., uric acid 10 mg. per 100 cc., creatinine 20 mg. per 100 cc. (present as creatinine zinc chloride), creatine 5 mg. per 100 cc., and acetoacetic acid 100 mg. per 100 cc.

The results given in Table II indicate that known amounts of phosphorus added to serum, the inorganic phosphorus content of which has been previously determined, may be quantitatively recovered.

Table III gives the results of the determination of the inorganic phosphorus in the sera of normal adults. The ages ranged from 20 to 35 years. The blood was removed between 9 and 11 a.m. and the serum separated within $1\frac{1}{2}$ hours. The determinations were performed within 36 hours of the time the blood was obtained.

Table IV gives the results of the determination of the inorganic phosphorus in sera of infants who showed no clinical evidences of rickets.

TABLE IV.

Inorganic Phosphorus Content of Normal Infant Serum.

Serum.	Age.	Inorganic P per 100 cc.
	mos.	*mg.*
10	12	5.2
19	13	4.6
20	14	5.8
21	16	5.0
22	10	5.3
53	4	5.7
55	36	6.4
Average		5.4

DISCUSSION.

The use of a solution of strychnine molybdate for the precipitation of phosphorus was first used by Pouget and Chouchak (1). The reagent as devised by these authors had to be used immediately after its preparation. Subsequently, Kober and Egerer (2) modified and improved the reagent so that it "was stable and gave quantitative and constant results." Other modifications have been made by Bloor (3), Medinger (4), Kleinmann (5), and Embden (6).

The reduction of molybdic acid with the production of a blue color has been known for many years. Some time ago, Taylor and Miller (7) devised a method for the determination of small amounts of phosphorus which depended on the precipitation of ammonium phosphomolybdate with the subsequent determination of the amount of molybdenum present by its reduction with phenylhydrazine. Quite recently a method has been devised by Bell and Doisy (8) which depends on the selective reduction of

the molybdenum present as phosphomolybdic acid, in an excess of molybdic acid, with hydroquinone as the reducing agent. The present method depends on the precipitation of the phosphorus as strychnine phosphomolybdate, with the strychnine molybdate reagent as devised by Embden, and the reduction of the molybdenum present in the precipitate by means of potassium ferrocyanide and HCl.

The precipitation of the phosphorus takes place very rapidly. 5 minutes after the addition of the strychnine molybdate reagent over 95 per cent of the inorganic phosphorus present is precipitated, and in 10 minutes the precipitation is complete, as no increase is obtained if the mixture is allowed to stand for $\frac{1}{2}$ hour. The precipitate is slightly soluble in water, and if too much water is used for washing, erroneous results will be obtained. When the quantity of water indicated in this method is used it will be found that the amount of precipitate in solution in the second washing is so slight that it produces no appreciable error. Also the amount of the reagent left after the second washing is so small that if it is placed in a 100 cc. flask and potassium ferrocyanide and HCl added no perceptible green color can be observed. It is not necessary for the precipitate to be mixed with the added water to produce thorough washing.

The green color produced by the reduction of the molybdate is very stable and no special precautions are needed when it is being read in the colorimeter. If the solutions are allowed to stand a long time it will be found that the color tends to become more intense and of a bluish tint, this change taking place more rapidly in the weak solutions than in the strong ones. If a weak solution is read at 40 in the colorimeter with the standard at 20, in 3 hours time it will change to about 39 producing an error of $+2.5$ per cent, while a strong solution will change over night from 10.5 to about 11.0 producing an error of -5 per cent. No error will be obtained from this source if the reading is made during the first hour after the addition of the potassium ferrocyanide and HCl.

The amount of potassium ferrocyanide added may be varied slightly without effecting the result. The amount added, however, should be between 19 and 21 cc. The HCl should be added from a burette as a variation in the amount added effects the rapidity with which the color develops. The amount added

should be between 9.8 and 10.2 cc. It is also necessary that the volume of fluid in the known and unknown during the development of the color should be approximately equal.

It is of interest to observe the concentration of the inorganic phosphorus in normal human serum, as determined by various other methods. Greenwald (9) found the "acid-soluble" phosphorus to vary from 2 to 6 mg. of P per 100 cc. Marriott and Haessler (10) found from 1 to 3.5 mg. of inorganic phosphorus per 100 cc. and Bloor (11) 1.9 to 3.8 mg. of inorganic phosphorus per 100 cc. of normal adult serum. Feigl (12) found the "acid-soluble" phosphorus to be under 4 mg. per 100 cc. Two determinations of serum from normal adults are recorded by Bell and Doisy (8) who found 3.5 and 3.9 mg. of inorganic phosphorus per 100 cc. Howland and Kramer (13) report an average of 2.1 mg. of inorganic phosphorus per 100 cc. in the serum of normal adults and an average of 5.4 mg. in the serum of twelve non-rachitic infants. The presence of a high inorganic phosphorus content in the serum of non-rachitic infants reported in the present paper is in agreement with the high content, first recorded by these authors.

The inorganic phosphorus content of normal adult serum as determined by the present method is slightly higher than recorded by most observers. Since it is possible that some of the "unknown phosphoric acid combinations" as well as the inorganic phosphorus might be extracted with the trichloroacetic acid, duplicate determinations were performed using trichloroacetic acid and acid ammonium sulfate as the extracting agents. Identical results were obtained. The possible extraction of some of the "unknown phosphoric acid combinations" by the strongly acid strychnine molybdate reagent was also considered. The supernatant fluid after its separation from the precipitate was allowed to stand 24 to 48 hours. A definite precipitate was found in the fluid from the serum while a barely perceptible amount was present in the fluid from the standard. It is highly improbable that this definite precipitate would have formed in the fluid from the serum if the "undetermined phosphoric acid" had been previously extracted.

The method as described above was devised by the author for the study of the inorganic phosphorus content of the serum in various clinical and experimental conditions. The principle of the

method, however, is applicable to the determination of the acid-soluble and lipoid phosphates but as the experiments being undertaken at the present time include the estimation of only the inorganic phosphorus content of serum, the details for the determination of the acid-soluble and lipoid phosphates have not been worked out.

CONCLUSIONS.

1. A rapid colorimeter method for the determination of the inorganic phosphorus in 1 cc. of serum has been described.

2. The presence of Na, K, Ca, Mg, dextrose, urea, uric acid, acetoacetic acid, creatinine, and creatine in the concentrations found in normal and pathological sera does not interfere with the determination of phosphorus by the technique described.

3. The results obtained on serum are accurate to within $\pm$ 5 per cent of the amount of inorganic phosphorus actually present.

4. Known amounts of phosphorus (as KH_2PO_4) added to serum may be recovered quantitatively.

5. The inorganic phosphorus content of the serum of normal adults is singularly constant, the variation in nineteen out of twenty-two cases being only from 3.5 to 4.0 mg. per 100 cc. The serum of normal infants has a much higher inorganic phosphorus content than is present in normal adults.

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13. Howland, J., and Kramer, B., *Am. J. Dis. Child.*, 1921, xxii, 105.

VITAMINE STUDIES.

IX. THE INFLUENCE OF THE DIET OF THE COW UPON THE QUANTITY OF VITAMINES A AND B IN THE MILK.*

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The suggestion that Funk[1] offered in 1913 that there was probably a definite relationship between the amount of vitamine secreted in the milk and that ingested in the food has become a well established fact. Since then many investigators have referred to this relationship, but it is only recently that any have carried out experiments with laboratory animals to prove it definitely. Without attempting to review completely the work done in this field attention may be called to a few important investigations.

In 1916, McCollum, Simmonds, and Pitz[2] stated that the two factors, vitamines A and B, pass into the milk only as they are present in the diet of the mother. These investigators observed the ability of the female rat to rear its young when confined to rations known to be adequate for growth when sufficient amounts of vitamines A and B are added, and inadequate for growth when either of these factors is omitted. They had no means of determining whether the omission of either vitamine A or B affected the amount of milk secreted by the rat and, therefore, whether the young received enough milk to permit normal growth. In

* Published with the approval of the Director as Paper No. 285, Journal Series, Minnesota Agricultural Experiment Station.

[1] Funk, C., *Biochem. J.*, 1913, vii, 211.

[2] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.

1918, Steenbock, Boutwell, and Kent[3] noting the variations in the amount of vitamine present in butter fat surmised that perhaps the rations on which the butter fat had been produced might be at fault. However, they further state that the ration is not the only factor to be considered as the butter fat produced by a cow fed exclusively on alfalfa hay was found to contain no demonstrable amounts of vitamine. They do not comment upon the fact that the butter had been kept in an unsalted condition in a poorly iced refrigerator for about 3 weeks and that mold had developed upon the surface; nor do they make any statement in regard to the quality of the alfalfa used. In 1920, Hess and Unger[4] suggested that an insufficiency of vitamine A in milk might be due to the winter fodder of the cow.

In 1919, Barnes and Hume[5] and Dutcher, Pierson, and Biester[6] announced simultaneously that they had noticed seasonal variations in the antiscorbutic properties of cow's milk. Experimental proof of this variation was later published by Hart, Steenbock, and Ellis,[7] Dutcher and coworkers,[8] and Hess, Unger, and Supplee.[9] Inasmuch as each of these investigators used a somewhat different method for studying this variation, the conclusion seems justified that the vitamine C content of cow's milk can be influenced materially by the food of the cow. However, Hughes, Fitch, and Cave[10] have recently reported that the milks of cows fed a vitamine-rich ration and a vitamine-poor ration showed no difference as to vitamine C, but that both vitamines A and B were decidedly greater in the milk from a vitamine-rich ration. The same was true of the butter made from the milk produced on these two rations.

[3] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

[4] Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.

[5] Barnes, R. E., and Hume, E. M., *Biochem. J.*, 1919, xiii, 306.

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[7] Hart, E. B., Steenbock, H., and Ellis, N. R., *J. Biol. Chem.*, 1920, xlii, 383.

[8] Dutcher, R. A., Eckles, C. H., Dahle, C. D., Mead, S. W., and Schaefer, O. G., *J. Biol. Chem.*, 1920–21, xlv, 119.

[9] Hess, A. F., Unger, L. J., and Supplee, G. C., *J. Biol. Chem.*, 1920–21, xlv, 229.

[10] Hughes, J. S., Fitch, J. B., and Cave, H. W., *J. Biol. Chem.*, 1921, xlvi, p. l.

Attention has already been called to the work carried on in this laboratory in regard to the variation in the vitamine C content of milk due to the food of the cow. The experiments which were being carried out at that time offered an excellent opportunity for the study of vitamines A and B of milk produced under favorable and unfavorable conditions. Up to this time only vitamine B, as it existed in the milk, had been studied, all investigations of vitamine A being made on the butter which had been separated from the milk.

The work of Hopkins and Osborne and Mendel, the most notable experiments in which milk is used as a source of vitamine B, will be discussed later on with the experiments which embody this paper.

EXPERIMENTAL.

The experiments described in this paper are the first, in so far as we are aware, in which both vitamine A and vitamine B of cow's milk have been quantitatively studied with respect to the changes which may occur in the milk due to a change from pasture to dry feed. In order to study such changes the amount of milk fed must necessarily be the minimum which would produce satisfactory growth. To find this minimum, experiments were begun in December, 1919, with the mixed milk from a Holstein and a Jersey cow which were on a vitamine-poor ration and whose milk was being used in another experiment.[8] Although at that time the actual details in carrying out the experiment differed somewhat from those which were finally adopted the results of these earlier experiments check very satisfactorily with those reported in this paper.

The plan first followed was that used by many investigators; *i.e.*, to start with a small amount of milk to furnish the vitamine which had been omitted from the ration and gradually to increase the milk until an amount was being given which just gave satisfactory results. 5 cc. were arbitrarily chosen as the minimum amount. This method of procedure was not suited for our work as the rats lost in weight very rapidly on the low vitamine ration and their condition became so precarious that they did not respond to small additions of the milk which was probably becoming poorer in vitamines each day. From these results it was decided that

10 cc. daily were the minimum amount of milk which would furnish adequate amounts of vitamines A and B, but in order to be certain of the outcome duplicate groups of rats were started which received 15 cc. of milk daily.

The milk used in these experiments was chosen to represent two types of milk: that produced by cows fed a ration presumably adequate in all respects, and that produced by cows fed a ration known to be deficient in vitamines but adequate in protein, mineral matter, and energy value. The ration fed the former group, which was the Station herd and includes Holstein, Jersey, Guernsey, and Ayrshire cows, was made up as follows:

	Parts.
Corn	200
Bran	200
Oats	200
Oil meal	140

Roughage was fed in the form of alfalfa hay and corn silage. During the summer months when the cows were on pasture this ration was continued but fed in smaller amounts than in the winter months. The ration which was fed to the latter group, which included two Holstein cows, consisted of equal parts of ground oats, ground barley, wheat middlings, and gluten feed with timothy hay and oat straw for roughage. This roughage was used for the first 2 months of the experiment, when the timothy hay was taken out as it was thought that it might be furnishing considerable amounts of vitamines to the ration. This ration is undoubtedly poor in vitamine A and furnishes inadequate amounts of vitamine B, and is a poorer ration than is fed during the winter months on the dairy farms producing the milk for large cities. During the period this ration was fed a fair flow of milk was maintained. The butter fat of the milk averaged 3.4 per cent which increased to 3.8 per cent after the cows had been on pasture for 3 weeks. These cows were placed on the vitamine-poor ration October 1, 1920, and continued on it until April 28, 1921, when the ration was changed to that of the Station herd. On May 16, 1921, the cows were turned out to pasture, the dry feed, however, being continued. The cows were milked morning and evening and samples of the mixed milk were taken for the feeding experiments.

The time for starting the rats on their experimental rations was so chosen that the milk used would be representative of a milk produced in midsummer when all conditions of feeding and pasture were good; in fall when the pasture was beginning to fail; in midwinter when the cows had to depend entirely on grain mixtures, silage, and hays; and in late winter and early spring when the effect of the change from winter feed to summer feed could be noted.

The rats used in this experiment were healthy, normal rats selected from our breeding colony. As far as possible five or six rats weighing from 65 to 75 gm. each were selected for each group. These were kept in separate cages so that a record could be kept of their food intake. The usual laboratory precautions were taken in regard to the sanitary conditions of the cages, drinking bottles, and feeding dishes. The rats themselves were kept free of animal parasites by the application of pine oil.[11]

The milk in 10 or 15 cc. portions, as the conditions of the experiment called for, was given to each rat the first thing in the morning and after the first few weeks of the experiment had passed the milk was consumed within a few hours, at least before it became sour. A little difficulty was experienced at first in getting the young rats to drink all of their milk but this difficulty did not extend over a sufficiently long period to affect the results of the experiment.

The basal ration used in our experiments was as free from vitamines as it is possible to make such a ration under the present conditions of our knowledge as to the nature of vitamines. The casein, which would be the most objectionable constituent in a vitamine-free ration because of adsorbed impurities, was made according to a method which is used in this laboratory and which we believe gives a very pure product.[12] The preparation of vitamine B might carry small amounts of vitamine A. However, inasmuch as the wheat embryo, used as a source of vitamine B, was first thoroughly extracted with ether, the amount of this vitamine in the subsequent alcoholic extract would doubtless be very small as it has been reported[13] that ether removes vitamine

[11] Kennedy, C., *Science*, 1921, liii, 364.

[12] Palmer, L. S., and Kennedy, C., *J. Biol. Chem.*, 1921, xlvi, 571.

[13] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549. Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xlii, 131.

A from plant tissue. Butter, melted at a low temperature and filtered, was used as the source of vitamine A. The salt mixture used in these rations was that given by McCollum.[14] The dextrin was prepared by autoclaving commercial starch, acidified with 0.2 per cent citric acid, at a pressure of 15 pounds for 6 hours. This was then dried and ground.

The basal ration used in this work was composed of purified casein 18 parts, salt mixture 3.7, agar 2, and dextrin to make up the 100 parts. When the milk used was to furnish the vitamine A the dextrin of the basal ration carried the alcoholic extract of 15 gm. of wheat embryo per 100 gm. of ration, and when the milk was to furnish the vitamine B, the basal ration carried 5 per cent of filtered butter fat. When the milk furnished both vitamines the basal ration with no additions was used.

Group I, Charts I and II.—This group comprised the rats fed summer milk; that is, the milk produced by cows which were feeding in the pasture. These cows were not in the Station herd but were the two, a Jersey and a Holstein, which had been used during the previous winter in the preliminary experimental work having received the vitamine-poor ration given above. The cows had been turned into pasture and, in addition, given the herd ration on May 27, 1920, and their milk was not used for experimental feeding until July 1, 1920. The condition of the pasture was excellent at the time and continued to be so until the early part of August when the weather became very hot and dry for a period of approximately 3 or 4 weeks. Rains began again in September and restored the pastures which remained in good condition through October. The first hard frost came November 2, 1920. It was thought, therefore, that the milk was representative of good quality summer milk. The drying of the pasture during August influenced the vitamine content of the milk, especially the vitamine B, but this effect was not apparent until 3 or 4 weeks later (see Chart I, Curves 180 to 183). This would indicate that the cow can secrete vitamines A and B into her milk for some time after the vitamine supply in her feed has been diminished, the vitamine content of her milk being kept up at the expense of her tissues and that when vitamines are again supplied

[14] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 123.

by her food they go first to her tissue and then to her milk. This postulate is further supported by the fact that the experimental animals do not respond immediately to the milk when the cows are changed abruptly from a vitamine-poor ration to one which is relatively rich.

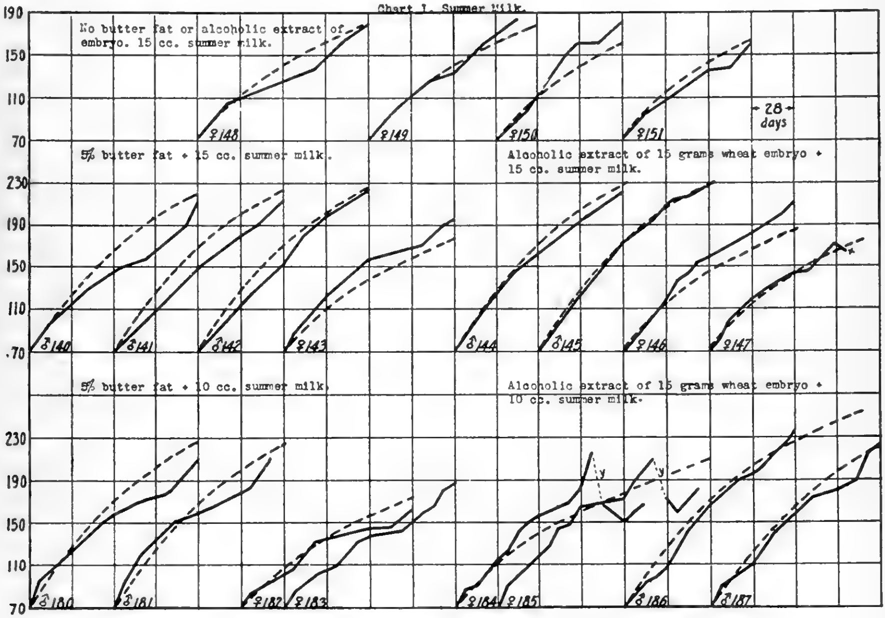

CHART I. Rats 140 to 151 inclusive, and Rats 180 to 187 inclusive were placed on the experimental ration July 1, 1920. The milk for these rats was supplied by the two cows which had been on a vitamine-poor ration from Jan. 17 to May 27, 1920, when they were turned out to pasture and, in addition, given the herd ration. Inasmuch as 4 weeks intervened between their going into pasture and the time when their milk was used for the experiment it was considered that the milk was representative of good quality summer milk. The effect of the drying of the pasture during August is apparent in the growth curves a few weeks later and is especially noticeable in the curves of the rats which were receiving their supply of vitamine B from the milk. This does not, however, demonstrate that vitamine B is diminished to a greater extent in the milk than vitamine A as the quantitative need of the rat for both vitamines has not yet been determined. Rats 148 to 151 inclusive which received both vitamines A and B from 15 cc. of this milk made normal growth which shows that the amount of each vitamine contained in 15 cc. of this milk is sufficient for normal growth.

The rats which were fed the milk produced in the fall months are included in this group due to the fact that for the first 2 months of the feeding period the cows were still on pasture. The fall milk was from the Station herd. There is no outstanding difference in the fall milk curves, Chart II, and the summer milk curves.

Chart II. Fall Milk.

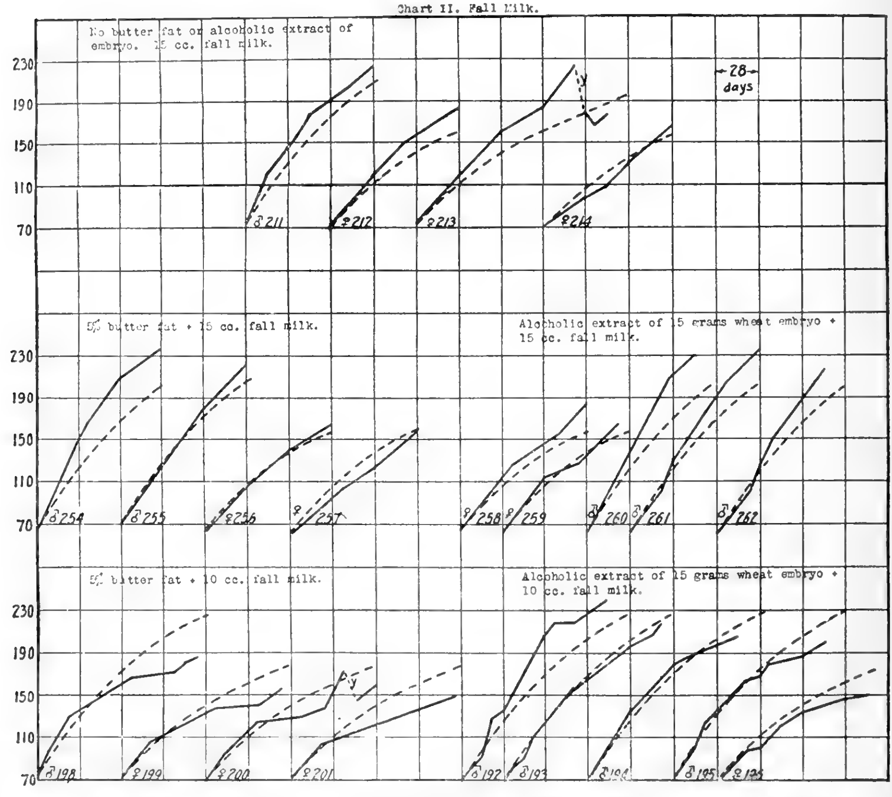

Chart II. Rats 192 to 201 inclusive, Rats 254 to 262 inclusive, and Rats 211 to 214 inclusive were placed on the experimental ration Sept. 1, 1920. The milk used for these rats was supplied by the Experiment Station herd. During September and October the pastures were green and the grass abundant. The first hard frost came Nov. 2 when the cows were removed from the pasture. The curves for Rats 198 to 201 inclusive show that the amount of vitamine B in 10 cc. of this milk is inadequate to support normal growth after 6 to 8 weeks. Comparison of these curves with those of Rats 304 to 307 inclusive (Chart VI) shows that the failure to make normal growth is not due to the fact that 10 cc. of milk will not furnish enough vitamine B but that 10 cc. of milk from cows feeding on poor pasture is not adequate.

The summer milk curves fall away from the normal from the effects of drying of pasture, and the fall milk curves start to fall off when the pasture season ends.

Group II, Charts III, IV, and V.—This group includes the rats fed the winter milk produced by the two Holstein cows. These cows had been placed on the vitamine-poor ration in October, 1920, and the feeding of this milk to the rats began in December. This afforded 2 months in which the cows could get rid of the vitamines in their tissues. However, it could not be determined whether the milk was becoming poorer each day in vitamines and would finally become so depleted that it would no longer cause

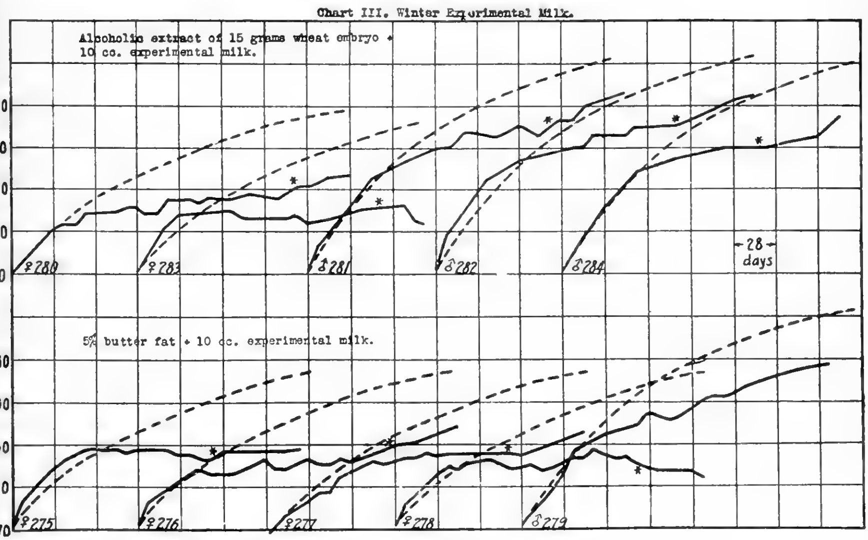

Chart III. Rats 275 to 284 inclusive were placed on the experimental ration Nov. 30, 1920. The milk used for these rats was supplied by the two Holstein cows which had been on vitamine-poor rations since the first of October. 10 cc. of this milk do not furnish adequate amounts of either vitamine A or vitamine B. Rats 275 to 279 inclusive became very thin and nervous during the course of the experiment and two rats, Nos. 275 and 278, died of lung infection near the end of the experiment. Although Rats 280 to 284 inclusive suffered severely in their growth from a lack of vitamine A, none showed signs of eye trouble indicative of a deficiency of this vitamine. May 1, 1921, the ration of the two experimental cows was changed to the herd ration and on May 16 the cows were turned out to pasture. This point is indicated by an asterisk on each curve.

Chart IV. Winter Experimental Milk.

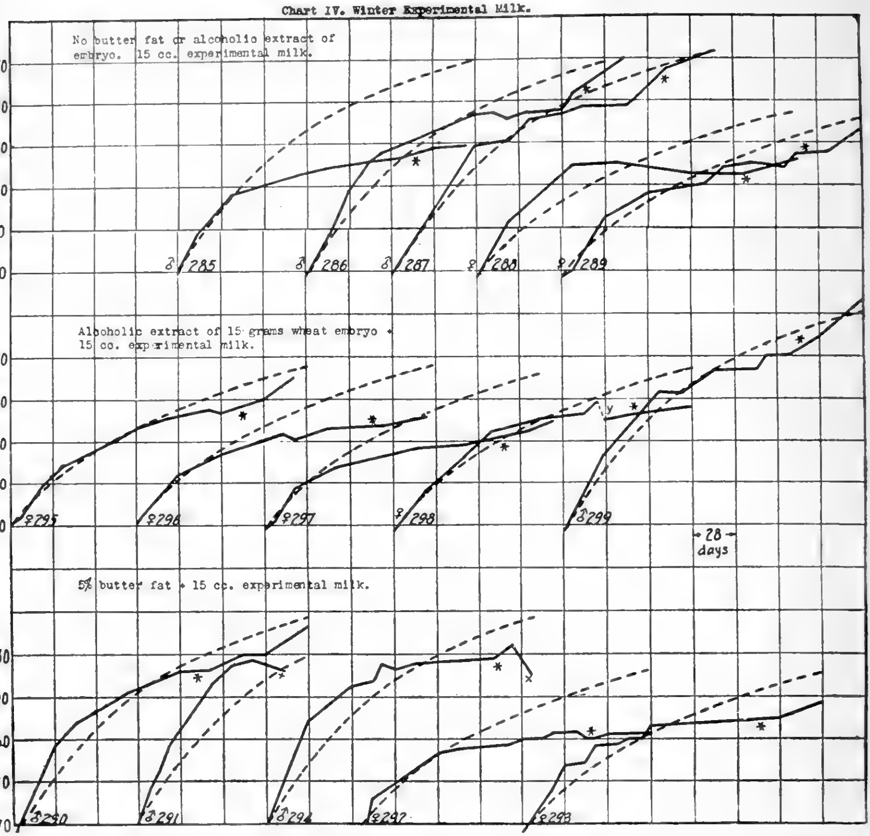

Chart IV. Rats 285 to 299 inclusive were placed on the experimental ration at the same time as the rats in Chart III; the only difference in their ration being that they received 15 cc. of milk instead of 10 cc. The increased amount of milk had a decidedly beneficial effect on the rats' growth curves. Although the increase in weight was normal or better than normal for a considerably longer period there was quite a long period when the rats were only able to maintain their weights. A comparison of this chart with Chart VI shows the great superiority of the quality of vitamine A contained in 10 cc. of winter milk from cows fed on an adequate ration over that contained in 15 cc. of milk from cows fed a ration known to be deficient in vitamines. The curves for Rats 285 to 289 inclusive show a greater divergence from the normal than do the other curves, but this is to be expected as there are two deficient factors in the ration of these rats while in the ration of the other rats there is but one deficient factor. Rats 291 and 294 died from an unknown cause. The cows were turned out to pasture at the point marked by the asterisk.

growth, or whether it was as deficient at this time as it ever would be, but still contained enough vitamine to cause some growth. 10 cc. of this milk do not furnish adequate amounts of either vitamines A or B after the first few weeks of feeding. The weights of the rats which received vitamine A and those which received vitamine B from 10 cc. of this milk began to fall away from the normal at about the same time although there is a slight extension of time in the case of the rats which received vitamine A from the

Chart V. Late Winter and Early Spring Experimental Milk.

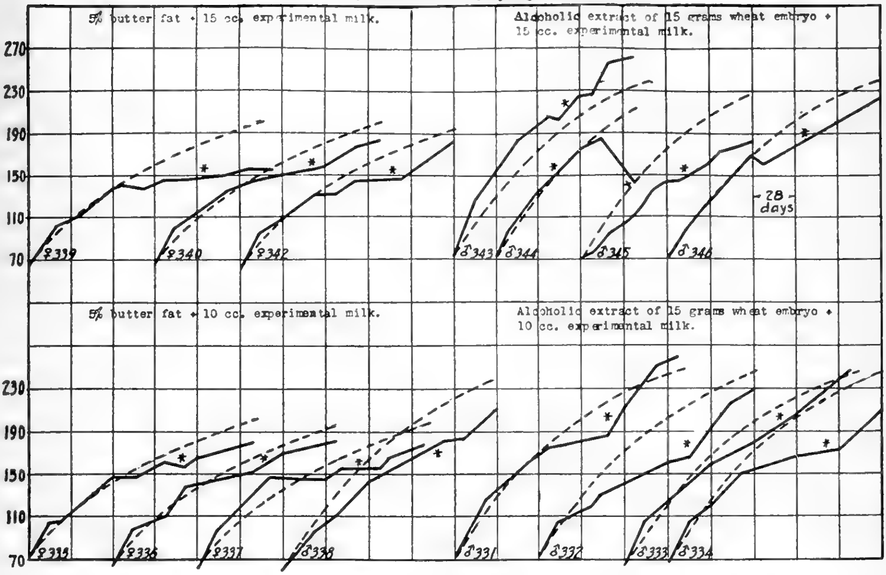

Chart V. Rats 331 to 346 inclusive were placed on the experimental ration Feb. 1, 1921. This group was started at this time to ascertain if the milk of the cows became progressively poorer in vitamines A and B as the length of time of feeding a vitamine-poor ration increased. Comparing this chart with Chart III, it is seen, in each group, that the rats stopped gaining weight at approximately 6 to 8 weeks after the beginning of the experiment. The curves in this chart (Chart V) appear better than those of Chart III because the rats were not on the experimental ration for as long a period, and, therefore, do not become as depleted. They also show a greater improvement after the cow's ration improves. These curves indicate, though they do not demonstrate, that if the vitamines are stored in the body of the cow it is only for a short time. The curves of the group receiving 15 cc. are only slightly better than those receiving 10 cc. Rat 344 died from an unknown cause.

milk. It is interesting to note that the male rats withstood the vitamine deficiency much better than the female rats. During the periods of the experiment although the rats which received vitamine A from 10 cc. of milk became thin, they had no xerophthalmia.

Further proof of the paucity of vitamine A in this milk is shown in Chart IX. The rats whose curves appear in this chart obtained their supply of vitamine A from butter that had been made from cream separated from this milk, during the last 4 weeks of the experiment, when the milk was undoubtedly very poor in vitamines. The butter was placed in small containers under an atmosphere of CO_2 and stored in an ordinary refrigerator, the temperature of which was approximately 10°C. Two groups of rats were fed purified rations carrying in one case 5 per cent of this butter

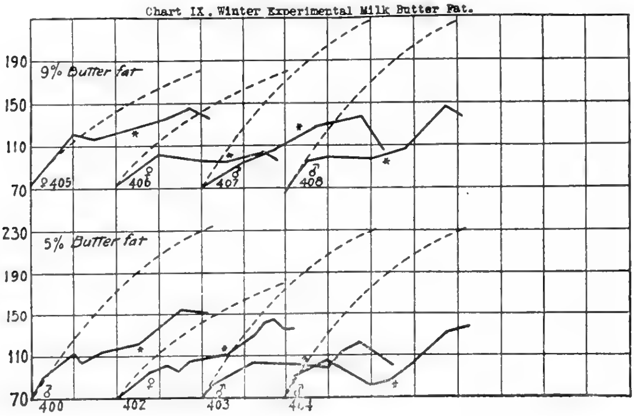

Chart IX. Rats 400 to 408 inclusive were placed on the experimental ration in the winter of 1921. The basal ration carried the alcoholic extract of 15 gm. of wheat embryo and in the case of Rats 400 to 404 inclusive, 5 per cent of butter fat made from the vitamine-poor milk, and in the case of Rats 405 to 408 inclusive, 9 per cent of the same butter fat. The asterisk indicates the point at which the fat in the ration of Rats 400 to 404 inclusive was increased to 15 per cent, and in the ration of Rats 405 to 408 inclusive to 20 per cent. That the rats did not improve very materially in this last group seemed to be due to the fact that the rats did not eat well of the ration because of its excessively greasy nature. Improvement in the former group took place on increase of the fat from 5 to 15 per cent but the curves never coincided with the normal. In all of our experimental rations 5 per cent of good butter fat has carried sufficient amounts of vitamine A for normal growth.

fat and in the other case 9 per cent. When the rats had ceased to grow the butter fat in each ration was increased to 15 and 20 per cent respectively. This increase gave only slightly better results. We believe that this deficiency is due to no other cause than to a lack of vitamine A in the ration of the cows as extreme care was exercised both in the separation and churning of the cream and in the subsequent storage of the butter.

The rats receiving their vitamine B from 10 cc. of milk became very nervous and timid and two, Rats 275 and 278, died of lung infections. In other groups where there was a deficiency of this vitamine the same lung infection occurred. None of the rats in this group showed a quick or marked improvement in their weights after the cows had gone to pasture. Whether these rats were in too depleted a condition to improve after the cows had gone to pasture, or whether the cows had become so depleted in vitamines A and B that 6 weeks was not a long enough period to show an improvement in the vitamine content of their milk, could not be definitely determined, although the rats of Chart V which had received the experimental milk for a shorter period and were in better physical condition showed a more decided upward trend in their curves. Perhaps the most striking inference which can be drawn from a comparison of Charts III and IV with Chart V is in connection with the observation that the rats on comparable rations grew to about the same weight before the vitamine deficiency became evident. As pointed out previously, there is no evidence to indicate that the milk fed to the rats of Chart V was any poorer in vitamine at the beginning of the experiment than that received by the rats in Charts III and IV, although the experiments labeled "Winter Experimental Milk" began 2 months before those designated "Late Winter and Early Spring Experimental Milk." As a matter of fact, the indications are rather that the quantitative deficiencies of the milk with respect to the vitamines were no greater in the late winter than several months earlier. The inference which we can draw from these data is either that the actual weight of vitamines required for growing rats of 70 to 150 gm. weight, a period of very active growth, is less than in larger rats, or that a certain period has been reached in the life cycle of the rat when the demand for vitamine is greatest. That this period is that of adolescence is indicated by the fact that

the rats in the experiment began to show signs of vitamine deficiency at the age when this species of animal attains sexual maturity.

The rats which received 15 cc. of this same winter milk maintained a normal growth curve for a considerably longer period than those which received 10 cc. but there was a long period when the rats were only able to maintain their weights.

Group III, Charts VI and VII.—The milk used for this group was the winter milk from the Station herd. The rats receiving 10 cc. of milk daily made remarkably good growth; in fact, it equalled that of those receiving 15 cc. This is of interest in view of the fact that Osborne and Mendel[15] have reported that 16 cc. of whole summer milk were necessary for satisfactory growth with a ration that was supplying the vitamine A. A comparison of Chart VI with Chart IV shows the great superiority of the quality of vitamine A contained in 10 cc. of the Station herd winter milk over that contained in 15 cc. of the winter milk from the two experimental cows. 15 cc. of the latter milk contained 0.526 gm. of butter fat and were much less efficient for growth than 10 cc. of the former milk which contained only 0.392 gm. of butter fat.

From the excellent growth of these rats we can conclude that a winter ration for cows may be made perfectly satisfactory as far as vitamines A and B of the milk are concerned by the proper combination of grain and leafy foods.

It will be noted that throughout the experiment there were only a few of the rats that reproduced. While it would have been desirable to have had reproduction, it was not the aim of the experiment to find a quantity of milk upon which perfect growth, as measured by the ability of the rat to reproduce at normal intervals would be attained, but rather to ascertain the effect of the cows fed on the quality and quantity of the vitamine produced in her milk. In order to obtain quantitative results in feeding it was, under the laboratory conditions at the time of the experiment, almost impossible to allow the rats to be together for more than a very short time each day and it is very difficult to obtain normal reproduction under such conditions. There were five females, however, which gave birth to young and reared them successfully.

[15] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537.

Attention may be called to the work of Hopkins[16] on the stimulating effect on growth of 2 cc. of cow's milk as an addendum to an artificial ration consisting of casein, fats, starch, sugar, and inorganic salts; and to his later work in which he substantiates this result. Because of these results it seemed necessary, after some of the preliminary experiments of this investigation had been completed and it was indicated that young rats could not grow on

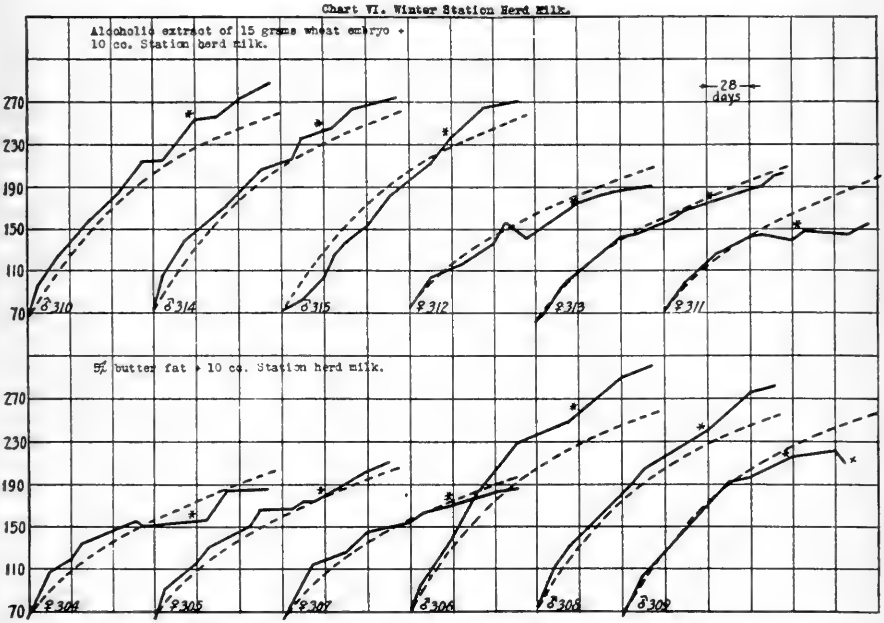

Chart VI. Rats 304 to 315 inclusive were started on the experimental ration Jan. 12, 1921. The milk fed to this group was furnished by the same herd that furnished the milk for the rats in Chart II. The rats receiving 10 cc. made remarkably good growth, in fact they equalled the growth of those receiving 15 cc. This fact demonstrates the value of an adequate combination of grain and leafy foods during the winter season. Rat 309 died of pneumonia. The time that the cows were turned out to pasture is indicated on the chart by an asterisk.

5 cc. of milk produced on a vitamine-poor ration, to further substantiate this result by repeating the experiment but to use a summer milk known to produce satisfactory growth when fed at

[16] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

a higher level. Therefore, two groups of rats were used: one group received, in addition to a ration which was complete in all respects except for its vitamine A, 5 cc. of milk to supply this vitamine; and a second group received, in addition to a ration which was complete in all respects except for its vitamine B, 5 cc. of the milk to supply this vitamine. The growth curves for these rats found in Chart VIII demonstrate very conclusively that 5 cc.

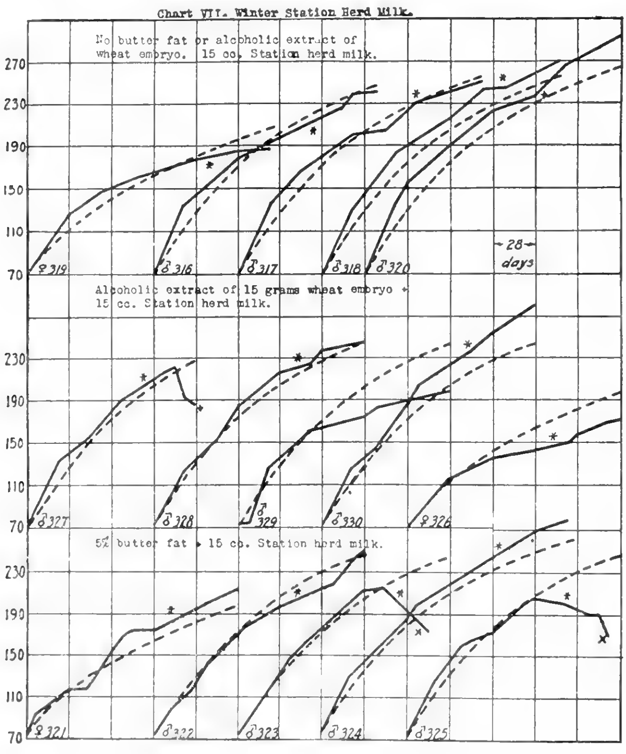

Chart VII. Rats 316 to 330 inclusive were started on the experimental ration Jan. 12, 1921, and received, daily, 15 cc. of milk from the Experiment Station herd. These rats evidently received sufficient vitamines from the start so that when the cows went into pasture any increase in the amount in their milk had little or no influence on the growth of the rats. That 15 cc. is ample to supply both vitamines A and B is shown by the curves for Rats 316 to 320 inclusive. Three rats, Nos. 323, 325, and 327, died from an unknown cause. The cows were turned out to pasture at the point marked by an asterisk.

of good quality summer milk will not promote normal growth in young rats. It is possible that the ration of the cows from which Hopkins obtained the milk he used was much richer in vitamines than that of the cows furnishing the milk for our experiment. That it is entirely a question of the ration of the cows seems to be clearly shown by the various results published by the different investigators. Hopkins has described carefully planned experiments in which he obtained satisfactory growth on a remarkably small amount of milk added to a purified ration. Osborne and Mendel[15] have described equally carefully planned experiments in which they state that "not until at least 16 cc. of fresh milk per

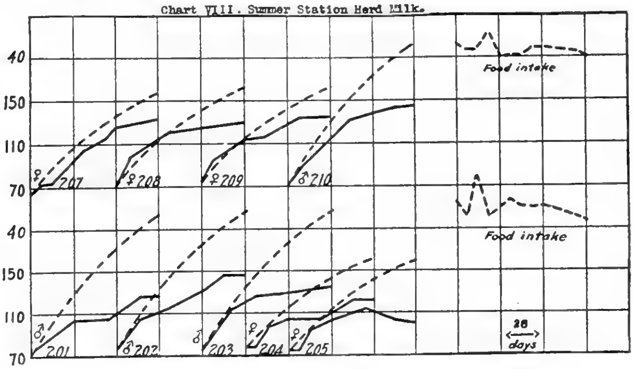

CHART VIII. Rats 201 to 210 inclusive were placed on the experimental ration in the summer of 1920. The milk used for these rats was the same as that used for the rats whose growth curves appear in Chart I. The failure to grow after the first 4 weeks is the result of a deficiency in vitamines A and B, owing to the small amount of milk (5 cc.) used as a source of these vitamines.

day were supplied along with the food mixture, was anything approaching a normal rate of growth secured. Even this amount sometimes failed." Later these investigators repeated this work using summer milk because they thought that the inferior quality of the milk, as a source of vitamine B, might be due to the winter ration of the cows, their earlier work having been carried out during the winter season when the cows were deprived of green pasture and were stall fed. In addition to the pasture grass, the cows were fed night and morning a ration consisting of corn gluten and

wheat bran together with hay and corn-stalks. Failure to grow became evident in approximately 25 days when the rats received 10 cc. of this milk, unpasteurized, from the beginning of the experiment. 15 cc. of the same milk barely sufficed as a source of vitamine B.

These results of Osborne and Mendel indicate that milk is a poor source of vitamine B. In our experience with milk we find that 10 and even 15 cc. of milk are inadequate to furnish either vitamines A or B when the milk is produced by cows which are feeding on a ration in which these vitamines are deficient, but that 10 cc. of milk are amply sufficient to furnish either vitamine provided the milk is produced by cows feeding on a ration which is adequate as to its vitamine content. It would, therefore, seem that milk becomes a good source of vitamine B when, in addition to feeding in pasture, the cow is given a grain and hay mixture rich in vitamines. Access to open pasture will not assure a ration rich in vitamines unless the pasture is always fresh and green. The feed in the pasture varies with the climatic conditions and in order to secure a milk uniform as to its vitamine content, it is necessary to give a good dry feed throughout the year. The unsatisfactory results obtained by the use of 15 cc. of summer milk, which Osborne and Mendel report, may be due to the fact that the dry feed ration of their cows was vitamine-poor and that practically the only source of vitamine B was pasture grass, a variable source.

That milk, as a source of vitamine B, may compare favorably with vegetable sources of this vitamine is shown in results which we have obtained in other work in this laboratory. We have found that while the alcoholic extract of 10 gm. of wheat embryo for each 100 gm. of ration will produce normal growth, this amount is not always dependable; therefore, it has been our custom, for some time past, to use the extract of 15 gm. of embryo. This extract, dried on 100 gm. of ration complete in all other respects, gives no better growth curves than 10 cc. of our Station herd winter milk when used as a source of vitamine B. This amount of milk adds to the ration of a rat 1.26 gm. of milk solids, which would not greatly enhance a complete ration unless the presence of vitamine C in the milk exerts a beneficial effect or that the milk contains some other food accessory which we have not as yet rec-

Chart X. Average Food Intake Per Week Per Rat.

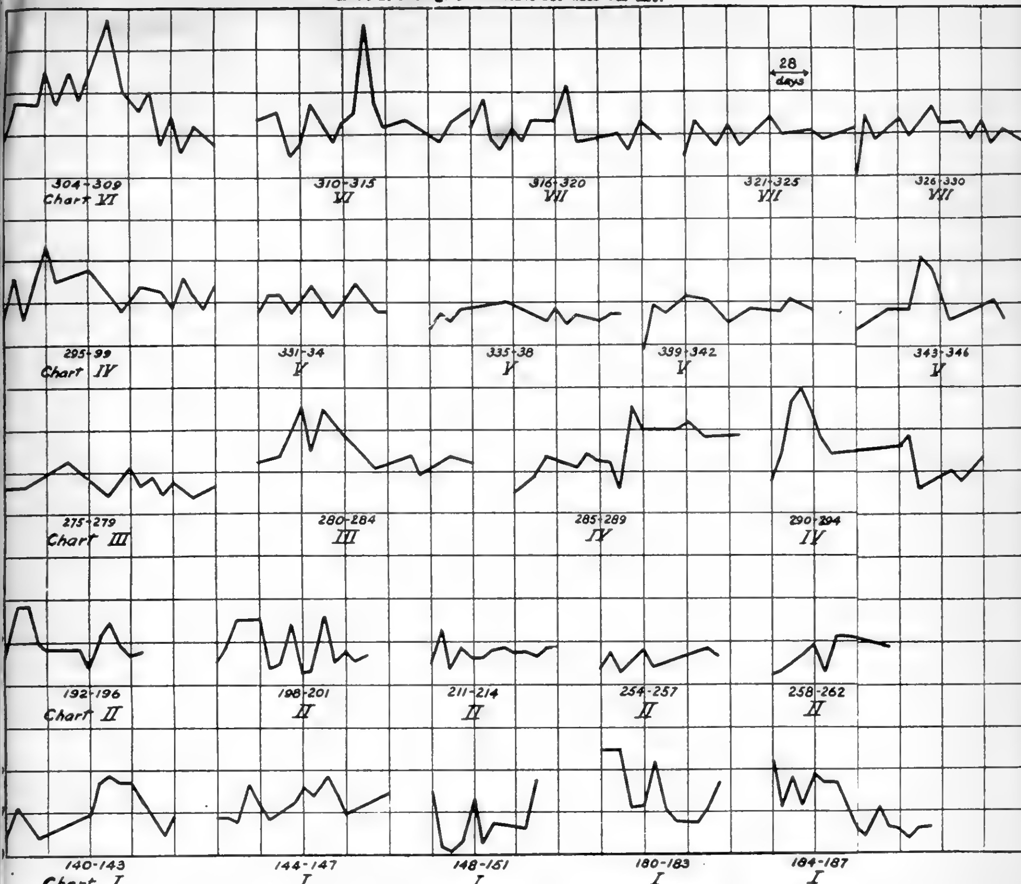

CHART X. The curves of this chart represent the average food intake per week per rat. Record the dietary intake of each rat was kept throughout the experimental period and the average of e records comprising each group was used in computing these graphs. A comparison of the curves ows that the food intake is approximately the same, with slight variations, whether the animal is owing or merely maintaining its weight, which fact does not agree with the generally accepted theory at the food intake corresponds very closely with increase in growth. The food intake of the rats ose growth curves are found in Charts III, IV, and V was approximately the same during the t 6 to 8 weeks of the experiment, when growth was normal, as during the remaining period of the periment when only maintenance of weight was accomplished. The actual food intake of the rats ose growth was excellent (Charts I, II, and VII) was no greater than that of the other groups harts III, IV, and V) which grew normally during only the first 6 to 8 weeks of the experiment d then merely maintained their weights. Moreover, the food intake for the rats of the former oups (Charts I, II, and VII) was less per gm. of body weight during the latter part of the experi-ental period than that of the rats of the latter groups (Charts III, IV, and V) during the same riod of the experiment. It would seem, therefore, that the effect of the vitamine is not necessarily e of appetite stimulation but rather a stimulation of metabolic processes which promote growth. The food intake for Rats 304 to 315 inclusive, Chart VI, does not parallel that of any of the other oups.

ognized. And again, baker's yeast which has recently come into great prominence as a rich source of vitamine B, has proved under the conditions of our experimentation, to be a less valuable source of vitamine B than we formerly supposed. We have found that when the yeast is mixed in the ration in the proportion of 10 gm. of yeast in each 100 gm. of ration that growth was not as good as when 10 cc. of our Station herd milk were used to supply the same vitamine. When the yeast was fed separately from the food mixture 0.6 gm. per day gave the same results as when the yeast was mixed in the ration in the proportion of 10 gm. to 100 gm. of ration. Osborne and Mendel[17] report that 15 cc. of summer milk are inferior to 0.2 gm. of brewer's yeast. We have found that the brewer's yeast, which we have been able to obtain, is much inferior in growth-promoting properties to baker's yeast.

SUMMARY.

Two types of milk, one produced on a ration typical of that used on some farms during the winter season and known to be deficient in its vitamine content, and a second representing that produced on a ration carrying ample amounts of vitamines A and B, have been used in this investigation. Each milk was fed so as to show in as nearly a quantitative manner as possible its content of vitamines A and B. Growth curves are given which show the possibility of growth on low and high levels of each milk. The importance of feeding the cow a ration adequate as to its vitamine content is demonstrated.

CONCLUSIONS.

1. The presence of vitamines A and B in cow's milk is entirely dependent upon their occurrence in the ration.

2. Stall fed cows will produce a milk rich in vitamines provided their ration consists of a proper combination of grains and leafy foods.

3. A vitamine-rich milk is not necessarily correlated with access to pasturage.

4. 10 cc. per day of either winter or summer milk is adequate to furnish either vitamine A or B to a rat provided the ration of the cow carries each in amounts adequate to meet her requirements.

[17] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*; 1920, xli, 515.

5. 5 cc. of the same milk that produced normal growth when used on a higher level does not furnish enough of either vitamines A or B to meet the requirements of growing rats.

6. The effect of the vitamine is not necessarily one of appetite stimulation but rather a stimulation of metabolic processes which promote growth.

In conclusion we wish to acknowledge the assistance of Mr. John W. Wilbur, formerly with the Division of Dairy Husbandry, in keeping careful oversight of the experimental cows whose milk was used in this investigation and in making weekly butter fat determinations on the milk.

STUDIES ON THE ACETONURIA PRODUCED BY DIETS CONTAINING LARGE AMOUNTS OF FAT.*

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The excretion of the acetone bodies—acetone, acetoacetic acid, and β-hydroxybutyric acid—in conditions in which the organism is not utilizing carbohydrate either through a deficiency of foodstuff of this kind in the diet or through the inability of the organism to metabolize the food when supplied, as in diabetes mellitus, has attracted attention for many years, and a large amount of literature has collected on the subject. In two papers recently published, Shaffer (1921, *a*, *b*) has summarized this literature, and has suggested certain methods of studying the problem which are somewhat different from those which have been used before. In this paper are reported some experiments which were carried out along the lines suggested, and which appear to support the theses advanced in these two articles.

In his first paper Shaffer (1921, *a*) reported experiments on the oxidation of mixtures of acetoacetic acid and glucose by alkaline hydrogen peroxide which showed that if there were present in the mixture one molecule or more of glucose for each molecule of acetoacetic acid, the acid was oxidized under suitable conditions of temperature, alkalinity, etc., but that if the relative concentration of glucose was less than this, the oxidation of the keto-acid was not as complete. In the second paper (Shaffer, 1921, *b*)

* A preliminary report of the clinical side of the work discussed was read before the meeting of the New York State Medical Association in Brooklyn, May, 1921, by Floyd R. Wright; a portion of the work formed part of a thesis presented for partial fulfilment of the requirement for the degree of Doctor of Philosophy at Washington University, St. Louis, in June, 1921, by Roger S. Hubbard.

he studied the problem from the point of view of the metabolism of human subjects, and concluded that a reaction of a similar nature takes place in the body. Since the appearance of these two articles Woodyatt (1921) has published a paper in which the subject is discussed from the standpoint of the practical treatment of diabetes, and in which data are presented which support the conclusions stated above.

The theory which has been developed in these papers, and on which the following paper is based, is that acetoacetic acid itself is not easily burned in the body, but that it forms with glucose, or with degradation products of glucose and related substances, a compound which is easily burned. To compounds which give rise, in the progress of metabolism, to acetoacetic acid the name "ketogenic" is given, while the name "antiketogenic" has been applied to compounds which furnish glucose or other related compounds with which the acetone body combines. The ketogenic compounds contained in the diets are the fatty acids contained in the fats and the α-amino-acids, leucine, tyrosine, phenylalanine, and possibly histidine which forms a part of the proteins. There is probably a molecule of the acetone bodies derived from each molecule of these compounds contained in the diet.

The amounts and source of the antiketogenic compounds contained in the diet are more uncertain. Glucose and related sugars, as levulose, form one source of these substances, whether taken as the sugars themselves or as the more complex carbohydrates. Protein yields glucose when fed to the total diabetic in amounts which vary with the different kinds of the foodstuff, and some percentage of the protein should therefore be included with the carbohydrate in figuring the total antiketogenic intake. There is, too, considerable data which indicate that glycerol yields glucose under some conditions, and so fat, from which glycerol is produced by hydrolysis in the organism must also be considered as a possible source of antiketogenic compounds.

The question is even more complicated than this, because it is not certain what derivatives of the glucose-forming α-amino-acids and glycerol act as antiketogenic compounds. Shaffer (1921, *b*) has pointed out this difficulty clearly. He states:[1]

[1] Shaffer (1921, *b*), p. 458.

". . . . the two carbon residues from glycocoll and the three carbon residues from the other sugar-forming amino-acids may have direct and immediate antiketogenic (ketolytic) action without condensation to glucose, and the same may be true of glycerol."

To determine the border-line diet which should just produce an excretion of the acetone bodies Shaffer (1921, *b*) calculated the molecular equivalents of the ketogenic compounds from fat and protein, and of the antiketogenic compounds from carbohydrate, protein, and the glycerol residue of the fat to the extent of the glucose which could be derived from them. From the analysis of the data so obtained he concluded that a diet containing 10 per cent of the calories in the form of protein, 10 per cent as carbohydrate, and 80 per cent as fat represented approximately the border-line diet. He studied this diet in the light of data contained in the literature and obtained experimentally, and showed that the theory was confirmed by such results as were available.

In the experiments reported in this paper an attempt was made to study this diet described by Shaffer, and diets in which the relative amounts of carbohydrate and fat were somewhat varied. To obtain a method of graphic representation of the various diets in terms of their "ketogenic balance" the following plan was adopted. The excess antiketogenic material derivable from protein, that is, the amount of glucose which protein would yield greater than that needed to bring about oxidation of the ketogenic material from the same protein, was calculated from the data presented by Shaffer (1921, *b*). He showed from the analyses of the α-amino-acid content of ox muscle given by Lusk (1917)[2] and from the glucogenetic power of protein, that there are twice as many gram molecules of antiketogenic substance (glucose) as of ketogenic compounds which can be derived from a given weight of this protein. Glucose is derived from ox muscle at the rate of 58 gm. for each 100 gm. of the protein ingested (Woodyatt, 1921), and there will be 29 gm. of excess glucose for each 100 gm. of this protein fed. The amounts of glucose and of acetoacetic acid which can be derived from different proteins vary, and 25 gm. have been chosen as a convenient average figure to express the amount of glucose available for additional antiketogenic

[2] Lusk (1917), p. 77.

action from 100 gm. of protein. This calculation is similar to that suggested by Woodyatt (1921). To the excess glucose from protein was added the glucose taken in the diet, and the sum was multiplied by 1.5 (molecular weight of glucose = 180; molecular weight of stearic acid = 284; of palmitic acid = 256; of oleic acid = 282; average = 270; $\frac{270}{180}$ = 1.5) to convert the result into terms of its fatty acid molecular equivalent. This product was divided by the fatty acid content of the diet (95 per cent of the fat) and the ratio was multiplied by 100 to give the resulting expression in the form of per cent.

This formula for expressing the "ketogenic balance" of any diet is expressed as follows:

$$100 \times \frac{1.5\ (\text{weight glucose} + 25\ \text{per cent weight protein})}{95\ \text{per cent weight fat}}$$

In preparing the charts in this paper the total carbohydrate content of the diet has been used instead of the glucose content. Such a substitution introduces an error, as the intake of starch in grams should be multiplied by 1.1 to give the correct amount of glucose to which it is equivalent, but the difference between the values is almost certainly within the limit of error, and the total carbohydrate content is more easily calculated from published tables.

Before proceeding to a study of the experiments, attention must be called to some of the limitations and advantages of the formula given above. In the first place it is based on an assumption which did not hold exactly for any of the diets studied. The formula assumes that all of the fat contained in the diets was fed in the form of glycerides of the higher fatty acids—palmitic, stearic, and oleic—and such a diet could not be fed for any considerable period. In one of the experiments an attempt was made to approximate such a composition for a few days, as will be described below, but for the most part butter and cream formed a large percentage of the fat ingested. These fats contain relatively large amounts (up to about 8 or 9 per cent) of their fatty acids in the form of compounds of comparatively low molecular weight, and, therefore, yield more ketogenic material per gram than do fats not derived from milk. In the formula a figure

lower than 1.5 should be used to convert antiketogenic compounds expressed as glucose into molecular equivalents of fatty acids when these fats are included in the diet. Butyric acid, for example, has a molecular weight of 88, and if tributyrin were the only fat fed the sum of the antiketogenic compounds expressed as glucose should be multiplied by 0.49 ($\frac{88}{180}$) to express the fraction in terms of relative molecular concentrations. However, this error will not change the numerical value of the expression by more than 5 per cent; the error introduced by figuring from the carbohydrate content of the diet was of the same order of magnitude, and the two should practically compensate for each other.

The second objection to the formula has been indicated already. It is impossible to be sure that the figure used to express the excess antiketogenic material from protein is correct. The value will vary for different proteins as their content of leucine, tyrosine, and phenylalanine varies, and will also vary because the glucogenic power of different proteins is different. The effect of this uncertainty upon the numerical expression is illustrated by the following figures. In a diet in which 10 per cent of the calories is fed as protein, 10 per cent as carbohydrate, and 80 per cent as fat the numerical value of the expression given above is 55 per cent. The values of similar expressions in which different figures express the excess glucose from protein would vary from 31 per cent if its antiketogenic power is neglected, to 71 per cent if its ketogenic power is neglected, and the glucose which can be derived from protein is figured at 60 per cent of the total weight. If protein contains both ketogenic and antiketogenic materials—and this is almost certainly the case—the different figures lie well within the limits of error with which such formulas can be applied to the study of actual diets.

In case the antiketogenic effect of protein depends, not on glucose, but on the two and three carbon atom residues derived from the sugar-forming α-amino-acids the values of the expression would be much higher than 71 per cent. In that case an α-amino-acid would figure three times as efficient as glucose if a two carbon atom residue takes part in the reaction as an antiketogenic compound, or twice as efficient if a three carbon atom residue takes such a part. It seems almost certain that it would

be possible to detect such a marked effect as this experimentally, and an attempt has been made to interpret the data presented below in such a way as to solve this problem.

In the formula given no account has been taken of the possible antiketogenic effect of the glycerol radicle present in the fats. This radicle is probably the most uncertain of the different possible sources of antiketogenic compounds contained in the diet, and the part which it plays in the reaction can be better discussed after a study of the data obtained experimentally. If different diets are fed, and the degree of acetonuria is noted and compared with some such numerical expression of their ketogenic balance as that suggested, the value of the ratio at which the excretion of the acetone bodies becomes normal will represent the condition of ketogenic antiketogenic equilibrium. From the numerical value of this ratio it should be possible to determine whether the glycerol residue figures as a source of antiketogenic compounds. If this residue yields glucose in the organism, and this glucose acts as an antiketogenic compound, enough of such material would be furnished in each gram of fat to combine with one-sixth of the ketogenic material, so that only five-sixths of the total fatty acids will be free to combine with the antiketogenic compounds from carbohydrate and protein; in this case acetonuria should develop and clear up when the diet has a value of 83 per cent. If glycerol in fat does not produce antiketogenic compounds in the organism, all of the fatty acid will take part in the reaction with antiketogenic material from other foods, and the border-line diet will have a numerical value of 100 per cent. If glycerol figures as an antiketogenic compound in the form of a three carbon atom residue, one-third of the total fatty acid will combine with it, two-thirds of it must be burned by the help of other foodstuffs, and the border-line diet should have a value of 67 per cent. In the experiments reported here an attempt has been made to make such comparisons, and to determine whether the glycerol residue of fat does possess an antiketogenic action.

The study of diets which produce border-line acetonuria and at the same time maintain the body weight of the subjects is rather difficult. The diets are markedly different from those generally eaten, and many patients return a portion of the fat untouched. There is also a temptation to break the dietary

restrictions not unlike the temptation to which diabetics are subject, and it would not be necessary for a patient to ingest much more carbohydrate than is furnished to spoil an experiment. In the series presented here only such cases are included as could be studied under rather close supervision in a department devoted exclusively to the study of nutritional diseases. We wish here to express our thanks to Dr. S. T. Nicholson, Jr., the director of this department, and the dietitians and nurses attached to it for their cooperation in our experimental work.

The series included two experiments on a normal subject, one of which has been previously reported in another connection, and studies on four cases of arthritis who were undergoing the dietary treatment recommended by Pemberton (1917) in which the carbohydrate intake is reduced. These cases can probably be considered as normals for the purpose of such a study, although Pemberton and Foster (1920) have stated that such patients show a slightly increased concentration of sugar in the blood and an abnormal rise in blood sugar after the administration of large doses of glucose.

In all of the experiments the attempt was made to furnish enough food to each patient to maintain the body weight unchanged. To accomplish this the basal metabolism was determined with the Benedict portable respiration calorimeter (Benedict, 1918) and enough calories were fed to allow for the maintenance of basal equilibrium and for the probable activity of the patient. Usually the food provided for a bed patient was so calculated as to furnish 20 to 25 per cent more calories than his basal requirement called for, and this was found to be satisfactory for most of the subjects. It is desirable that the patients should be in nitrogen as well as in metabolic equilibrium, and at the same time that the protein content of the diet should be kept low to diminish the uncertain factor of its part in the ketogenic expression. In the experiments reported 10 per cent of the total calories were fed as protein in most of the diets studied. The relationship between the nitrogen intake and the output of nitrogen in the urine was determined, and it was found that there was little difference between them. If the excess antiketogenic compound had been figured from the urinary nitrogen instead of from the protein intake, the value of the ratio described would

not have varied beyond the limits of experimental error. The intake of carbohydrate and of fat formed, respectively, the sources of 10 and of 80 per cent of the calories in the basal diet, and of varying percentages—5 and 85 per cent, 15 and 75, 20 and 70 per cent—in the other diets studied. An attempt was made to feed each of these diets for a period long enough to determine the level of acetone excretion which corresponded to it, but it was usually necessary to change the more severe diets before such an equilibrium was established.

The diets used were figured from the tables given in Joslin's Diabetic Manual (Joslin, 1919); they were prepared under the direction of a competent dietitian, and food not eaten was weighed, and the proper allowance made in the record; a complete sample diet is given for one of the cases. While a majority of the patients ate the diets as furnished, two did not, and the results of the studies carried out on them are accordingly not wholly satisfactory. It has seemed best to include these cases in this report, however, as they serve as a check upon the results obtained upon other subjects.

The urines were sent to the laboratory daily. It was impossible to control the completeness of the collection through creatinine determinations because the presence of the acetone bodies in urine interferes with the method of analysis (Morris, 1915), and all of the cases except one showed a large excretion of acetone on all of the more severe diets fed. This lack of suitable control of the accuracy of collection made it seem best to record and plot the concentration of the acetone bodies as well as their total excretion. In some specimens marked variations in volume, total nitrogen content, and ammonia nitrogen content almost certainly show failure to collect accurate 24 hour specimens.

These daily urines were analyzed for acetone bodies by a method recently described (Hubbard, 1921) by which the acetone plus acetoacetic acid were determined together as acetone, and the β-hydroxybutyric acid was determined separately, also as acetone. Total nitrogen was determined by the direct Nesslerization method of Folin and Denis (1916), slightly modified to permit the use of the oxidizing and Nessler's reagents, described by Folin and Wu (1919). Ammonia determinations were made by the permutit method of Folin and Bell (1917). In some instances

other factors were studied which were connected more indirectly with the main problem. Total acidity was determined in the urine of two of the patients by the method described in Folin's Manual (Folin, 1916),[3] and its hydrogen ion concentration in one of the experiments by a colorimetric method using the standard universal buffer solution described by Acree, Mellon, Avery, and Slagel (1921). This solution was standardized before use against phosphate solutions of known hydrogen ion concentration. Besides these determinations on the urine the stripped weight of the patients was recorded, and in most instances the tension of carbon dioxide in the alveolar air was estimated. This determination was carried out in Cases II and III by the method of Marriott (1916) and in the other cases by the Fridericia method (Fridericia, 1914; Poulton, 1915).

The results obtained are given in Tables I to VI, and plotted in Charts 1 to 6. In these charts the diet is indicated at the top in terms of both total food and of percentage of the calories furnished by the three main classes of foodstuffs. When the diet varied much from day to day the average intake was made the basis of this plot. The numerical value of the expression

$$100 \times \frac{1.5 \text{ (weight carbohydrate)} + 25 \text{ per cent (weight protein)}}{95 \text{ per cent weight fat}} = \text{N per cent}$$

was plotted to correspond with the intake of food for each day, and the daily excretion of all the acetone bodies reckoned as the sum of the acetone which could be formed from them was plotted below it. Since slight increases of acetone above normal may be of considerable importance in this study, the plan was adopted, in two of the cases studied, of plotting the excretion of acetone bodies in terms of their concentration in the urine upon paper with logarithmic characteristics. This method shows differences which are actually slight but which may figure as large percentage increases because the normal amounts are small. In these plots the two fractions of the acetone bodies—acetone plus acetoacetic acid and β-hydroxybutyric acid—were plotted separately to show the relationship between the two fractions, and both were plotted as acetone to make the curves comparable. The concentrations

[3] Folin (1916), p. 103.

instead of the total excretions were used because of occasional failure in the collection of accurate 24 hour specimens.

Cases I and IV are reports of two experiments carried out on the same normal subject (one of the authors, R. S. H.). The data reported under the heading "Case I" have been previously presented (Hubbard, 1921) and are repeated here because a comparison with those obtained on the same subject in a later experiment show some things which are not as well brought out in other studies. The subject was a man 5 ft. 10½ in. tall, who weighed 165 lbs. and who at the time of the first experiment was 28 years old. During both of the periods he did light laboratory work while the experiments were going on. The first series of results was obtained before the appearance of Shaffer's papers in 1921 and the diet was differently planned from those used in the other experiments. The fat and carbohydrate were fed in different relative amounts—multiples of 50 gm., as a study of the table shows—and an attempt was made to feed sufficient protein to keep the caloric intake constant. The subject had been living on a normal mixed diet up to the first day of the experiment. There was a slight negative nitrogen balance during the first part of it, and a slight positive one after the diet had become more nearly normal, but the difference was not great enough in either case to affect the calculation of the probable ketogenic balance seriously. The development and clearing up of acetonuria is clearly shown in Table I and Chart 1. There was certainly an increased acetonuria on a diet which contained 250 gm. of fat, 50 gm. of carbohydrate, and 68 gm. of protein; this diet has a ketogenic balance of 42 per cent in terms of the formula suggested for expressing that balance. The excretion of the acetone bodies was slightly increased during the first 3 days of the experiment, but the increases were so slight that they cannot be attributed with certainty to the diet. The acetonuria completely cleared up when a diet having a ketogenic balance of 152 per cent was fed, and it seems certain that the border-line diet, that is, the diet representing ketogenic equilibrium, must lie between the two extreme diets fed, and probably does not lie far from that fed at the start of the experiment which has a ketogenic balance of 97 per cent. Attention should be called to the gradual increase and decrease of acetonuria as it developed and cleared up; a study of the table and chart makes it seem improbable that the second diet was fed long enough to cause a maximum excretion of acetone to correspond with its composition.

The experiment recorded under the heading "Case IV" was carried out on the same subject as was that recorded under "Case I." The height and weight were approximately the same as those given in the preceding paragraph, and the age was 33 years. The basal metabolism measured 1,750 calories per day. The diets were calculated and fed as here described, and the subject ate the entire amount of every diet provided. The collection of urine samples was accurate. There was some loss of weight during the first part of the experiment, but when diets were fed which caused an

TABLE I.

Case I.

Date.	Diet.							Urine.						
	Protein.		Fat.		Carbohydrate.		Calories.	Volume.	Total N.	NH_4N	Acetone + diacetic.		β-hydroxybutyric acid.	
1917	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
Mar. 12	96	15	201	69.5	100	15.5	2,573	935	12.5	0.748	0.7	0.006	2.2	0.020
" 13	96	15	201	69.5	100	15.5	2,573	1,100	17.0	0.748	4.4	0.048	4.4	0.042
" 14	96	15	201	69.5	100	15.5	2,573	905	16.6	0.776	7.2	0.065	3.5	0.032
" 15	68	10.1	251	82.4	50	7.5	2,711	1,095	17.8	0.787	34.2	0.374	18.8	0.206
" 16	68	10.1	251	82.4	50	7.5	2,711	815	14.6	0.815	58.0	0.473	69.0	0.562
" 17	68	10.1	251	82.4	50	7.5	2,711	1,180	12.5	0.914	68.0	0.802	112	1.42
" 18	72	11.7	175	63.7	150	24.6	2,448	980	13.4	0.964	28.0	0.274	35.9	0.352
" 19	72	11.7	175	63.7	150	24.6	2,448	1,040	11.5	0.849	10.3	0.107	5.8	0.061
" 20	72	11.7	175	63.7	150	24.6	2,448	860	9.75	0.670	3.3	0.026	3.4	0.029
" 21	72	11.7	175	63.7	150	24.6	2,448	810	10.6	0.667	2.8	0.023	3.4	0.026
" 22	72	11.7	175	63.7	150	24.6	2,448	610	9.94	0.490	1.4	0.024	4.0	0.024

Results of the determinations of the acetone bodies are expressed in terms of acetone.

excretion of only very small amounts of acetone the weight was quite constant. There was a slightly negative nitrogen balance as based on the determination of urine nitrogen during the first part of the period of study, and a slight retention later when the diet fed was more nearly normal. The excretion of acetone increased from 40 mg. on the day before the experiment to about 1.25 gm. after 4 days on a diet which contained 10 per cent

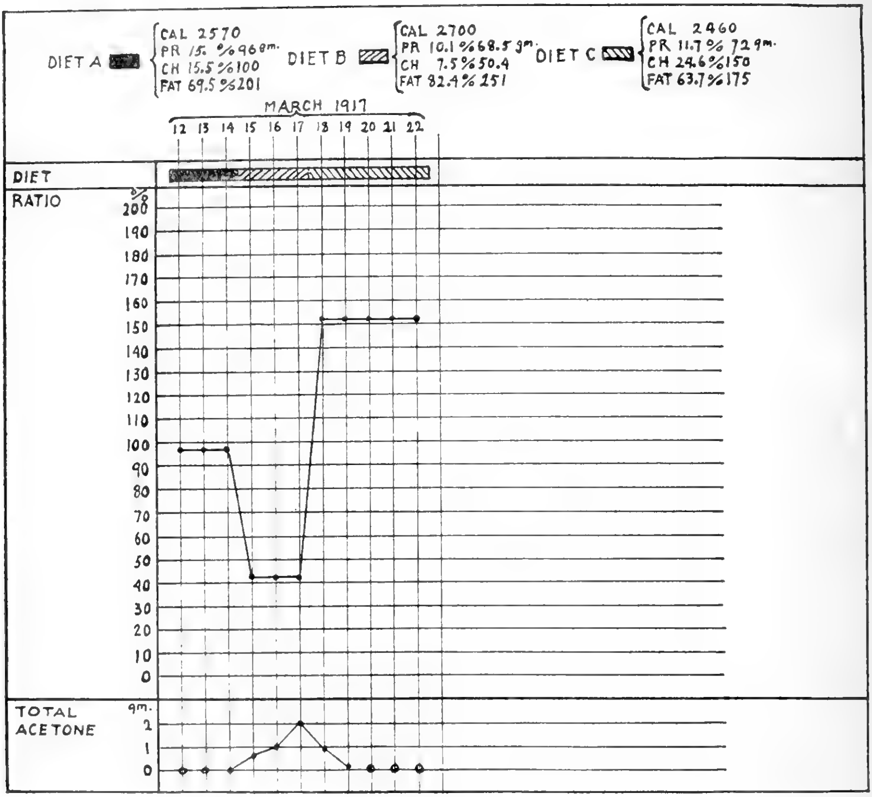


CHART 1.

of the calories as protein, 10 per cent as carbohydrate, and 80 per cent as fat; this increase makes it seem probable that there were more ketogenic than antiketogenic compounds in the diet. When the relative amount of carbohydrate in the diet was increased the excretion of the acetone bodies diminished, but did not return to the normal values when the diet contained 20 per cent of the calories as carbohydrate and 70 per cent as fat; oleomargarine and olive oil were substituted for the larger part of the butter fat in

this diet for 3 days, the 7th, 8th, and 9th of August, but the excretion of the acetone bodies was not measurably decreased further. However, the amounts of acetone excreted when this diet, which has a ketogenic balance of 108 per cent, was fed were not markedly different from those found on a less severe diet which had a ketogenic balance of 79 per cent, and were not very large in either case. The border-line of increased acetonuria appears to lie, for this case, between diets giving values of 78 and 108 per cent, although the value may be higher if the excretion of very small amounts of acetone is regarded as important in determining when ketogenic antiketogenic equilibrium has been established. It is noticeable, from comparing these two experiments on the same individual, that the excretion of acetone depends on the ratio between fat and carbohydrate rather than on the fat content of the diet.

Detailed Diet.

Case IV.

July 15.

Breakfast: Eggs, 2; Bacon, 20 gm.; Cream, 45 cc.; Butter, 15 gm.; Bread, 15 gm.; 10 per cent fruit, 70 gm.

Dinner: Meat, 60 gm.; 5 per cent vegetable, 135 gm.; Potato, 30 gm.; Cream, 40 cc.; Cheese, 15 gm.; Bread, 15 gm.; Butter, 25 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 135 gm.; Bacon, 10 gm.; Cream, 35 cc.; Cheese, 15 gm.; 10 per cent fruit, 50 gm.; Bread, 15 gm.; Butter, 27 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 50 cc. Forenoon—Olive oil, 30 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 16.

Breakfast: Eggs, 2; Bacon, 20 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 20 gm.; 10 per cent fruit, 80 gm.

Dinner: Meat, 60 gm.; Bread, 10 gm.; 5 per cent vegetable, 120 gm.; Potato, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.; Butter, 30 gm.; Watermelon, 45 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 120 gm.; Cheese, 15 gm.; Butter, 25 gm.; Bacon, 10 gm.; Cream, 60 cc.; Bread, 20 gm.; Watermelon, 45 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 20 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 17.

Breakfast: Eggs, 2; Bacon, 20 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 20 gm.; 10 per cent fruit, 80 gm.

Dinner: Meat, 60 gm.; Bread, 10 gm.; 5 per cent vegetable, 120 gm.; Potato, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.; Butter, 30 gm.; Watermelon, 45 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 120 gm.; Cheese, 15 gm.; Butter, 25 gm.; Bacon, 10 gm.; Cream, 60 cc.; Bread, 20 gm.; Watermelon, 45 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 20 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 18.

Breakfast: Eggs, 2; Bacon, 20 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 20 gm.; 10 per cent fruit, 80 gm.

Dinner: Meat, 60 gm.; Bread, 10 gm.; 5 per cent vegetable, 120 gm.; Potato, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.; Butter, 30 gm.; Watermelon, 45 gm.

Supper: Meat, 30 gm.; 5 per cent vegetable, 120 gm.; Cheese, 15 gm.; Butter, 25 gm.; Bacon, 10 gm.; Cream, 60 cc.; Bread, 20 gm.; Watermelon, 45 gm.

Extras (one-third to each meal): Olive oil, 90 cc.; Lemon, 20 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 19.

Breakfast: Eggs, 2; Bacon, 30 gm.; Bread, 10 gm.; Butter, 20 gm.; Cream, 30 cc.; Watermelon, 60 gm.

Dinner: Meat, 60 gm.; 5 per cent vegetable, 135 gm.; Cheese, 15 gm.; Butter, 34 gm.; Cream, 30 cc.; Watermelon, 60 gm.

Supper: Meat, 30 gm.; Bacon, 30 gm.; 5 per cent vegetable, 135 gm.; Cream, 30 cc.; Cheese, 15 gm.; Butter, 30 gm.; Watermelon, 60 gm.

Extras (one-third to each meal): Olive oil, 80 cc., Lemon, 15 cc.

Summary: Carbohydrate, 31.5 gm.; Protein, 63 gm.; Fat, 236 gm.

July 20.

Breakfast: Eggs, 2; Bacon, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Watermelon, 100 gm.

Dinner: Meat, 45 gm.; Cheese, 15 gm.; 5 per cent vegetable, 180 gm.; Butter, 30 gm.; Cream, 60 cc.; Watermelon, 65 gm.

Supper: Meat, 30 gm.; Bacon, 30 gm.; 5 per cent vegetable, 180 gm.; Cheese, 15 gm.; Butter, 27 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 31.5 gm.; Protein, 63 gm.; Fat, 236 gm.

July 21.

Breakfast: Eggs, 2; Bacon, 30 gm.; Butter, 25 gm.; Cream, 60 cc.; Watermelon, 100 gm.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 180 gm.; Cheese, 15 gm.; Butter, 30 gm.; Cream, 90 cc.; Watermelon, 65 gm.

Supper: Meat, 30 gm.; Bacon, 30 gm.; 5 per cent vegetable, 180 gm.; Cheese, 15 gm.; Butter, 27 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 31.5 gm.; Protein, 63 gm.; Fat, 236 gm.

July 22.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; Bread, 15 gm.; 10 per cent fruit, 40 gm.; Butter, 30 gm.; Cheese, 15 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Cheese, 15 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Butter, 25 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 23.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Cheese, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; Cream, 60 cc.; 5 per cent vegetable, 90 gm.; Butter, 25 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Cheese, 15 gm.

Extras (one-third to each meal): Olive oil, 80 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 242 gm.

July 24.

Breakfast: Eggs, 2; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.; Bacon, 30 gm.; 10 per cent fruit, 100 gm.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Cheese, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; Cream, 60 cc.; Butter, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Cheese, 15 gm.

Extras (one-third to each meal): Olive oil, 40 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 202 gm.

July 25.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Cheese, 15 gm.; Bread, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Cheese, 15 gm.; Bread, 20 gm.; Butter, 25 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 26.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 25 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 35 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 27.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; 5 per cent vegetable, 90 gm.; Cream, 45 cc.; Bacon, 20 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Cream, 45 cc.; Bread, 35 gm.; Butter, 30 gm.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 28.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 29.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Cream, 45 cc.; Butter, 30 cc.

Supper: Bacon, 40 gm.; 5 per cent vegetable, 90 gm.; Potato, 30 gm.; Bread, 35 gm.; Butter, 30 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

July 30.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; Cheese, 15 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; Cream, 60 cc.; Cheese, 15 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Butter, 25 gm.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

July 31.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; 5 per cent vegetable, 90 gm.; Cheese, 15 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Cream, 60 cc.; Butter, 30 gm.

Supper: Duck, 40 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; Cheese, 15 gm.; Bread, 20 gm.; Butter, 22 gm.; 10 per cent fruit, 55 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

Aug. 1.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 15 gm.; Butter, 20 gm.; Cream, 60 cc.

Dinner: Meat, 45 gm.; Cheese, 15 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 40 gm.; Bread, 15 gm.; Butter, 30 gm.; Cream, 60 cc.

Supper: Meat, 30 gm.; Cheese, 15 gm.; Bacon, 25 gm.; 5 per cent vegetable, 90 gm.; 10 per cent fruit, 55 gm.; Bread, 20 gm.; Butter, 25 gm.; Cream, 60 cc.

Extras (one-third to each meal): Olive oil, 60 cc.; Lemon, 15 cc.

Summary: Carbohydrate, 63 gm.; Protein, 63 gm.; Fat, 222 gm.

Aug. 2.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; 5 per cent vegetable, 60 gm.; Bread, 30 gm.; Bacon, 25 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 10 per cent fruit, 100 gm.; 5 per cent vegetable, 60 gm.; Bread, 30 gm.; Potato, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 3.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 4.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; Potato, 30 gm.; 5 per cent vegetable, 60 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 5.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 30 cc.; Cream, 90 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 6.

Breakfast: Egg, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 110 gm.; Bread, 30 gm.; Butter, 30 gm.; Milk, 60 cc.; Cream, 90 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 60 gm.; Potato, 30 gm.; 10 per cent fruit, 100 gm.; Bread, 30 gm.; Butter, 30 gm.; Cream, 90 cc.; Milk, 30 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 7.

Breakfast: Egg, 1; Egg white, 1; Bacon, 30 gm.; Oleo, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 200 gm.; Bread, 30 gm.; Milk, 60 cc.; Cream, 30 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; 5 per cent vegetable, 150 gm.; Potato, 30 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Cream, 20 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 5 per cent vegetable, 150 gm.; Potato, 30 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Milk, 30 cc.; Cream, 30 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 8.

Breakfast: Egg, 1; Egg white, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 200 gm.; Oleo, 30 gm.; Bread, 30 gm.; Cream, 30 cc.; Milk, 60 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; Potatoes, 30 gm.; 5 per cent vegetable, 150 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Cream, 30 cc.; Milk, 60 cc.

Supper: Egg, 1; Bacon, 30 gm.; 10 per cent fruit, 50 gm.; 5 per cent vegetable, 150 gm.; Oleo, 30 gm.; Potato, 30 gm.; Bread, 30 gm.; Milk, 30 cc.; Cream, 30 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 9.

Breakfast: Egg, 1; Egg white, 1; Bacon, 30 gm.; Oats, 10 gm.; 10 per cent fruit, 200 gm.; Bread, 30 gm.; Oleo, 30 gm.; Cream, 30 cc.; Milk, 60 cc.

Dinner: Meat, 30 gm.; Bacon, 25 gm.; Potato, 30 gm.; 5 per cent vegetable, 150 gm.; 10 per cent fruit, 50 gm.; Bread, 30 gm.; Oleo, 30 gm.; Milk, 60 cc.; Cream, 30 cc.

Supper: Egg, 1; Bacon, 30 gm.; 10 per cent fruit, 50 gm.; 5 per cent vegetable, 150 gm.; Oleo, 30 gm.; Bread, 30 gm.; Potato, 30 gm.; Milk, 30 cc.; Cream, 30 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 125 gm.; Protein, 63 gm.; Fat, 194 gm.

Aug. 10.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Potato, 30 gm.; Bacon, 20 gm.; 5 per cent vegetable, 90 gm.; Bread, 35 gm.; Butter, 25 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; Potato, 30 gm.; 5 per cent vegetable, 90 gm.; Bread, 35 gm.; Butter, 35 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

Aug. 11.

Breakfast: Eggs, 2; Bacon, 30 gm.; 10 per cent fruit, 150 gm.; Bread, 20 gm.; Butter, 30 gm.; Cream, 90 cc.

Dinner: Meat, 60 gm.; Potato, 30 gm.; Bacon, 20 gm.; 5 per cent vegetable, 150 gm.; Bread, 35 gm.; Butter, 25 gm.; Cream, 45 cc.

Supper: Bacon, 40 gm.; Potato, 30 gm.; 5 per cent vegetable, 90 gm.; Bread, 35 gm.; Butter, 35 gm.; Cream, 45 cc.

Extras (one-third to each meal): Olive oil, 30 cc.; Lemon, 10 cc.

Summary: Carbohydrate, 94 gm.; Protein, 63 gm.; Fat, 208 gm.

20 per cent cream was used. All food was weighed after cooking.

TABLE II.

Case IV.

Date.	Diet.							Weight.	Alveolar CO_2.	Urine.						
	Protein.		Fat.		Carbohydrate.		Calories.			Volume.	Total N.	NH_4 N	Acetone + diacetic.		β-hydroxybutyric acid	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*mm.*	*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
July 15	?	?	?	?	?	?	?	76.8		500	11.2	0.373	4.5	0.026	3.1	0.017
" 16	63	10	222	80	63	10	2,506	76.8	36	640	12.8	0.450	39.1	0.250	26.5	0.169
" 17	63	10	222	80	63	10	2,506	76.8	36	675	12.9	0.497	34.2	0.234	21.6	0.146
" 18	63	10	222	80	63	10	2,506	76.8		720	14.4	0.621	81.7	0.587	75.4	0.532
" 19	63	10	222	80	63	10	2,506		33	700	13.1	0.700	67.4	0.472	132	0.928
" 20	63	10	236	85	32	5	2,502		33	780	12.5	0.975	115	0.897	173	1.35
" 21	63	10	236	85	32	5	2,502	76.2	31	1,200	12.6	1.20	120	1.44	356	4.27
" 22	63	10	236	85	32	5	2,502	76.0	33	1,380	12.8	1.62	157	2.17	382	5.27
" 23	63	10	222	80	63	10	2,502	75.4	31	1,100	12.2	1.80	154	1.70	366	4.03
" 24	63	9.5	242	81	63	9.5	2,684		29	840	11.6	1.20	172	1.45	364	3.06
" 25	63	10.7	202	78.5	63	10.7	2,320			820		1.64	125	1.03	264	2.16
" 26	63	10	222	80	63	10	2,502	75.0	37	740	10.9	1.19	95.2	0.707	174	1.29
" 27	63	10	208	75	94	15	2,500		35	820	10.7	1.17	74.5	0.602	64.4	0.527
" 28	63	10	208	75	94	15	2,500	74.8	42	780	9.74	0.900	71.0	0.562	94.5	0.736
" 29	63	10	208	75	94	15	2,500		40	840	9.90	0.764	47.2	0.396	54.6	0.458
" 30	63	10	208	75	94	15	2,500		36	930	10.3	0.685	42.2	0.392	25.4	0.236
" 31	63	10	222	80	63	10	2,502	76.3	43	755	9.20	0.605	43.9	0.332	43.9	0.332
Aug. 1	63	10	222	80	63	10	2,502		40	780	9.77	0.600	117	0.917	70.3	0.542
" 2	63	10	222	80	63	10	2,502	76.0	41	830	10.4	0.602	78.0	0.608	110	0.914
" 3	63	10	194	70	125	20	2,498		40	925	10.1	0.514	64.1	0.594	60.8	0.561
" 4	63	10	194	70	125	20	2,498	76.3	41	1,060	9.48	0.623	46.0	0.495	78.2	0.827

Aug. 5	63	10	194	70	125	20	2,498	76.1		900	9.68	0.530	36.1	0.324	32.9	0.296
" 6	63	10	194	70	125	20	2,498	75.8	41	820			34.5	0.283	45.3	0.327
" 7	63	10	194	70	125	20	2,498	75.7	40	800	8.70	0.534	10.0	0.080	11.8	0.094
" 8	63	10	194	70	125	20	2,498		40	850	9.15	0.512	20.0	0.162	10.8	0.092
" 9	63	10	194	70	125	20	2,498	75.8	40	830	9.22	0.452	23.0	0.191	43.2	0.358
" 10	63	10	194	70	125	20	2,498		42	800	10.0	0.488	20.0	0.160	21.6	0.172
" 11	63	10	208	75	94	15	2,500			800	10.7	0.574	16.7	0.134	13.3	0.107
" 12	63	10	208	75	94	15	2,500	75.8		860	12.3	0.640	20.6	0.229	33.6	0.288

Results of determinations of the acetone bodies are expressed in terms of acetone. The subject upon whom this experiment was performed was the same as the one used for Experiment 1.

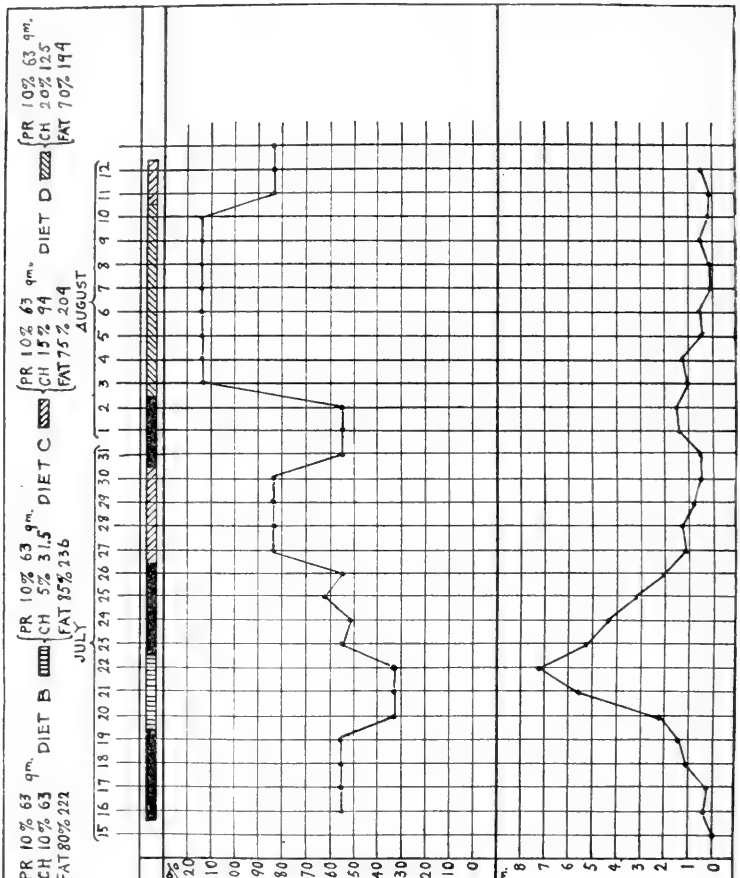
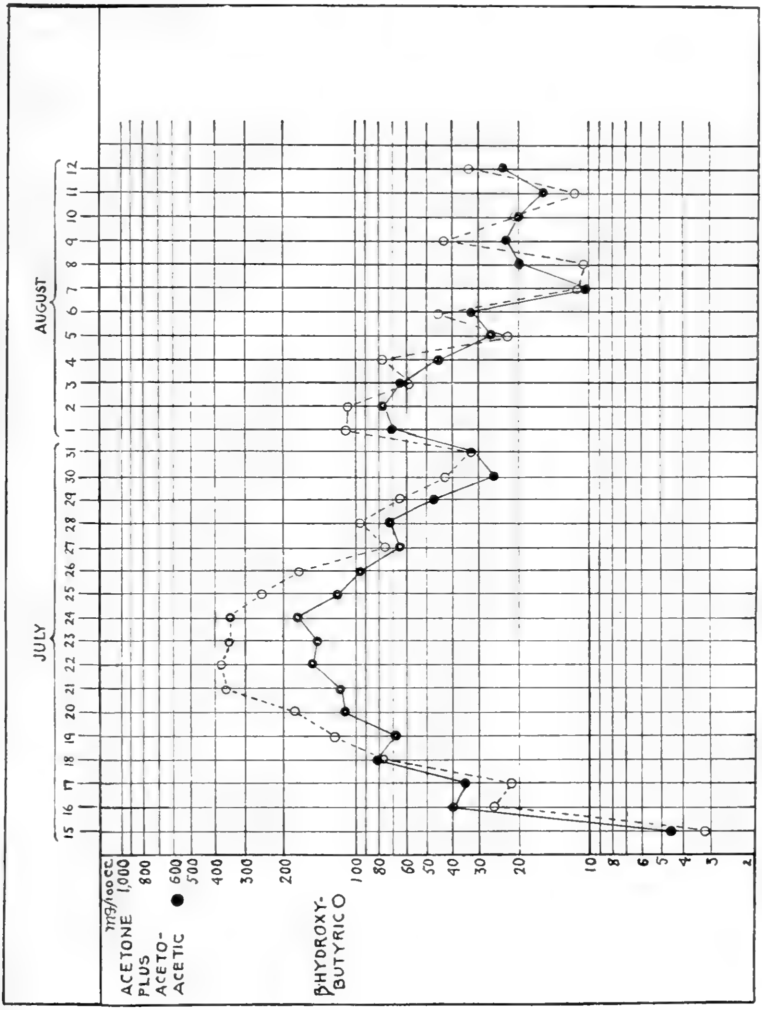

DIET A PR 10% 63 gm. CH 10% 63 FAT 80% 222
DIET B PR 10% 63 gm. CH 5% 31.5 FAT 85% 236
DIET C PR 10% 63 gm. CH 15% 94 FAT 75% 204
DIET D PR 10% 63 gm. CH 20% 125 FAT 70% 194
JULY
AUGUST
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 1 2 3 4 5 6 7 8 9 10 11 12
DIET
RATIO
%
120 110 100 90 80 70 60 50 40 30 20 10 0
TOTAL ACETONE
gm.
8 7 6 5 4 3 2 1 0

CHART 2.



JULY
AUGUST
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 1 2 3 4 5 6 7 8 9 10 11 12
mg/100cc
1,000 800 600 500 400 300 200 100 80 60 50 40 30 20 10 8 6 5 4 3 2
ACETONE PLUS ACETO-ACETIC ●
β-HYDROXY-BUTYRIC ○

CHART 2A.

TABLE III.

Case II.

Date.	Diet.							Weight.	Urine.								
	Protein.		Fat.		Carbohydrate.		Calories.		Volume.	Reaction.	Acid.	Total N.	NH_3 N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	gm.	per cent	gm.	per cent	gm.	per cent		kg.	cc.	pH	cc. 0.1 N	gm	gm.	mg./100 cc.	gm.	mg./100 cc.	gm.
Feb. 16	?	?	?	?	?	?	?		1,570	6.0	358		0.295	0.2	0.007	0.5	0.009
" 17	36	9.5	133	81	36	9.5	1,478	68.5	1,730	5.2	299		0.250	0.8	0.013	0.7	0.013
" 18	36	9.5	133	81	36	9.5	1,478	68.0	1,760	5.75	226		0.226	3.7	0.067	2.5	0.044
" 19	36	9.5	133	81	36	9.5	1,478	67.7	2,140	5.3	361		0.306	6.6	0.142	5.1	0.110
" 20	36	9.5	133	81	36	9.5	1,478										
" 21	36	9.5	133	81	36	9.5	1,478	67.2	1,795	5.3	301		0.346	9.5	0.171	6.1	0.112
" 22	36	9.5	133	81	36	9.5	1,478	67.0	2,190	5.3	316	5.10	0.353	6.5	0.144	4.7	0.085
" 23	36	9.5	133	81	36	9.5	1,478	67.0	1,880	5.35	276		0.246	11.3	0.136	12.1	0.155
" 24	36	9.5	133	81	36	9.5	1,478	67.0	1,575	5.4	290		0.303	6.9	0.109	7.6	0.121
" 25	36	9.5	133	81	36	9.5	1,478	67.1	1,400	5.5	291	6.07	0.334	15.5	0.210	22.6	0.329
" 26	36	9.5	133	81	36	9.5	1,478	66.8	1,380	5.35	264	7.07	0.307	12.5	0.183	13.2	0.183
" 27	36	10	126	79.5	37	10.4	1,426	66.7									
" 28	36	10	126	79.5	37	10.4	1,426	66.4	1,240	5.4	253		0.285	7.8	0.104	8.6	0.096
Mar. 1	36	10	126	79.5	37	10.4	1,426	66.4	1,565	5.3	289	7.10	0.284	4.1	0.064	4.7	0.074
" 2	36	10	126	79.5	37	10.4	1,426	66.3	1,300	5.7	224	6.50	0.192	5.2	0.068	2.9	0.038
" 3	36	10	126	79.5	37	10.4	1,426	66.2	1,220	5.4	284		0.200	5.5	0.066	4.7	0.058
" 4	45	10	153	75.1	68	15	1,816	66.2	1,460	5.5	246		0.225	4.1	0.060	2.3	0.034
" 5	45	10	153	75.1	68	15	1,816										
" 6	18		90		59		1,122	66.0									
" 7	45	10	153	75.1	68	15	1,816	66.2									
" 8	45	10	153	75.1	68	15	1,816	66.2	1,395	5.3	280		0.269	1.7	0.024	2.0	0.028
" 9	45	10	153	75.1	68	15	1,816	66.2	1,300	5.45	260		0.260	2.2	0.028	2.3	0.030
" 10	45	10	153	75.1	68	15	1,816	66.2	1,385	5.45	276		0.281	1.0	0.014	1.0	0.013

Results of determinations on the acetone bodies are expressed in terms of acetone.

Case II was a woman, Miss A. G., aged 47 years, whose height was 5 ft. 2 in. and who weighed 137 lbs. Her basal metabolism measured 1,335 calories per day. Like all of the pathological cases included in the series, she was a severe chronic arthritic of long standing. She made every effort to cooperate, but after a little more than a week she found it difficult to take the basal diet, and it was accordingly modified as shown in Table III and Chart 3. The diet fed during the first part of the experiment did not contain enough food to maintain the weight of the patient, and there was a progressive loss during the first 2 weeks. In four out of five determina-

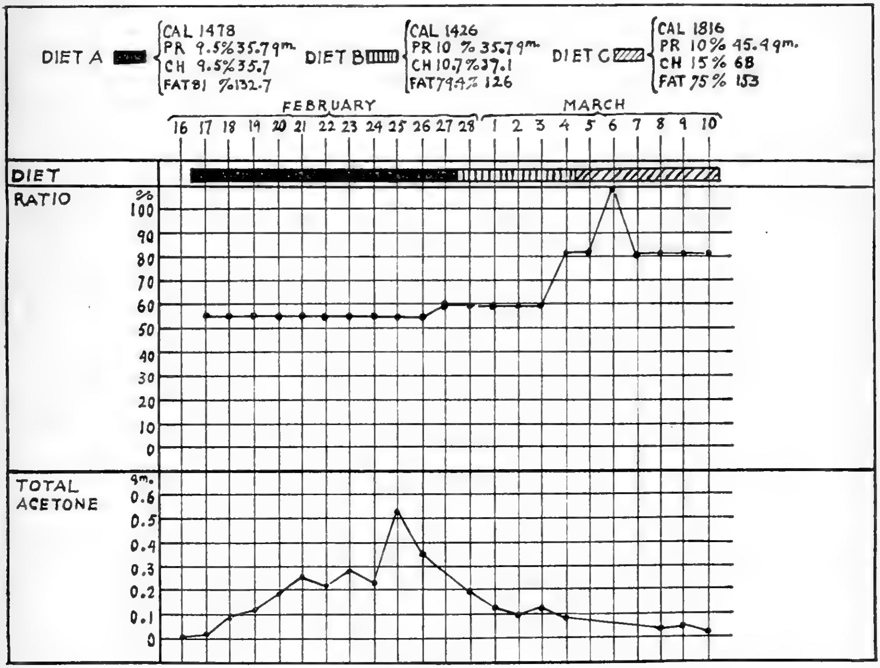


CHART 3.

tions of urinary nitrogen made during this period there was more found than was contained in the food taken, but on the day when the difference was largest, Mar. 1, the ratio used to express the ketogenic balance had a value of 56 per cent when calculated from the food fed and of 59 per cent when the protein burned was calculated from the urinary nitrogen; this difference is almost certainly within the limits of experimental error.

This case showed the smallest excretion of the acetone bodies of any in the series (the scale which has been used in plotting the acetone excretion is ten times as great as it is in the other cases), but still, when diets approximating the 10, 10, 80 per cent, diet were fed, the concentrations of

TABLE IV.

Case III.

Date.	Diet.								Weight.	Alveolar CO_2.	Urine.								
	Protein.		Fat.		Carbohydrate.		Calories.	$NaHCO_3$			Volume.	Reaction.	Acid.	Total N.	NH_4N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*gm.*	*kg.*	*mm.*	*cc.*	*pH*	*cc. 0.1N*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
Mar. 11	?	?	?	?	?	?	?		40.1		815	5.7	280	8.15	0.610	3.2	0.026	2.0	0.016
" 12	37	10	134	80	37	10	1,502		40.1	34	550	5.4	260	6.90	0.526	16.7	0.092	24.5	0.135
" 13	37	10	134	80	37	10	1,502			34	510	5.5	322	7.55	0.425	44.3	0.226	142	0.725
" 14	37	10	134	80	37	10	1,502		40.2	33	580	5.35	306	6.40	0.596	73.2	0.417	206	1.19
" 15	37	10	134	80	37	10	1,502		40.0	34	690	5.3	265	6.13	0.616	59.3	0.408	163	1.13
" 16	37	10	134	80	37	10	1,502	1.94	40.0	34	420	5.55	219	4.42	0.700	113	0.475	402	1.69
" 17	37	10	134	80	37	10	1,502	1.94	40.0	35	895	5.6	244	8.72	0.793	59.2	0.530	156	1.40
" 18	37	10	134	80	37	10	1,502	1.94	40.2	30	640	5.85	128	4.42	0.505	103	0.613	265	1.69
" 19	37	10	134	80	37	10	1,502	3.89	40.0	34	815	6.05	222	7.24	0.469	64.8	0.528	116	0.945
" 20	37	10	134	80	37	10	1,502	3.89		34	575	6.6	117	6.39	0.288	108	0.620	276	1.59
" 21?	37	10	134	80	37	10	1,502	3.89	40.2	37	340	6.3	130	4.45	0.219	90.7	0.308	209	0.713
" 22	37	10	134	80	37	10	1,502	3.89	40.4	37	675	6.3	199	7.20	0.374	88.2	0.595	165	1.12
" 23?	37	10	134	80	37	10	1,502		40.0	37	350	6.0	148	5.38	0.326	99.7	0.349	264	0.925
" 24	37	10	134	80	37	10	1,502		40.4	33	800	5.5	282	6.66	0.547	57.4	0.459	114	0.914
" 25	37	10	134	80	37	10	1,502		40.0		730	5.7	198	5.42	0.456	46.3	0.338	95.6	0.698
" 26?	37	10	141	85	19	5	1,493		40.2	30	320	5.7	153	3.66	0.426	88.9	0.284	209	0.670
" 27?	37	10	141	85	19	5	1,493			27	380	5.7	176	5.42	0.574	209	0.458	290	1.10
" 28	37	10	141	85	19	5	1,493		40.0	31	685	5.7	307	9.78	0.976	168	1.15	568	3.88

Mar. 29	37	10	134	80	37	10	1,502			28	820	5.6	296	7.46	1.32	103	0.851	315	2.58
" 30	37	10	134	80	37	10	1,502			30	840	5.55	255	7.48	1.10	74.3	0.625	187	1.57
" 31	37	10	134	80	37	10	1,502			31	760	5.7	231	5.44	0.760	49.2	0.374	87.5	0.666
Apr. 1	37	10	134	80	37	10	1,502				510	5.9	192	6.81	0.574	74.2	0.378	143	0.798
" 2	37	10	125	75	56	15	1,495				960	5.9	231	6.10	0.514	25.9	0.248	26.4	0.254
" 3	37	10	125	75	56	15	1,495			32	840	6.2	168	6.70	0.457	10.0	0.084	6.9	0.058
" 4	37	10	125	75	56	15	1,495			31	325	5.85	197	4.62	0.358	21.0	0.068	35.5	0.115
" 5	37	10	125	75	56	15	1,495			29	555	5.84	244	5.84	0.347	21.8	0.121	26.6	0.147
" 6	37	10	125	75	56	15	1,495			33	595	5.8	238	5.67	0.356	20.7	0.123	30.0	0.178
" 7	37	9.5	141	81	37	9.5	1,565			33	570	5.85	246	5.32	0.518	31.5	0.178	46.7	0.266
" 8?	37	9.5	141	81	37	9.5	1,565			33	320	5.7	179	4.00	0.320	80.4	0.257	217	0.695
" 9	37	9.5	141	81	37	9.5	1,565			35	620	5.55	303	6.20	0.620	93.2	0.573	256	1.59
" 10	37	10	117	70	75	20	1,501			36	860	5.55	324	5.50	0.384	22.5	0.190	30.7	0.263
" 11	37	10	117	70	75	20	1,501			35	825	5.9	250	5.33	0.292	16.2	0.133	17.7	0.145
" 12	37	10	117	70	75	20	1,501			36	740	6.25	207	5.09	0.284	6.7	0.050	4.4	0.033
" 13	37	10	117	70	75	20	1,501			36	715	6.1	211	4.98	0.286	9.0	0.063	7.7	0.050
" 14	37	10	117	70	75	20	1,501			36	520	6.2	192	4.42	0.260	8.6	0.015	6.4	0.033
" 15	37	10	117	70	75	20	1,501		40.9										

Results of determinations of the acetone bodies are expressed in terms of acetone.

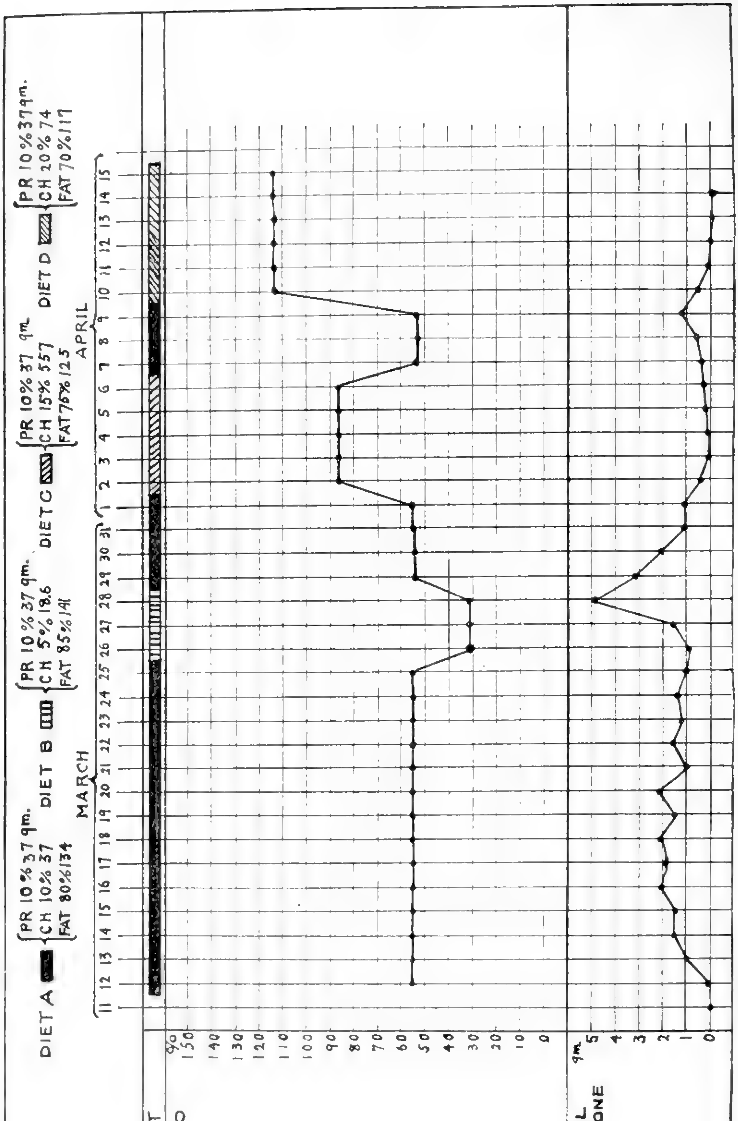

DIET A
PR 10% 37 gm.
CH 10% 37
FAT 80% 134
DIET B
PR 10% 37 gm.
CH 5% 18.6
FAT 85% 141
DIET C
PR 10% 37 gm.
CH 15% 55.7
FAT 75% 125
DIET D
PR 10% 37 gm.
CH 20% 74
FAT 70% 117
MARCH
APRIL
DIET
RATIO
TOTAL ACETONE
gm.

CHART 4.

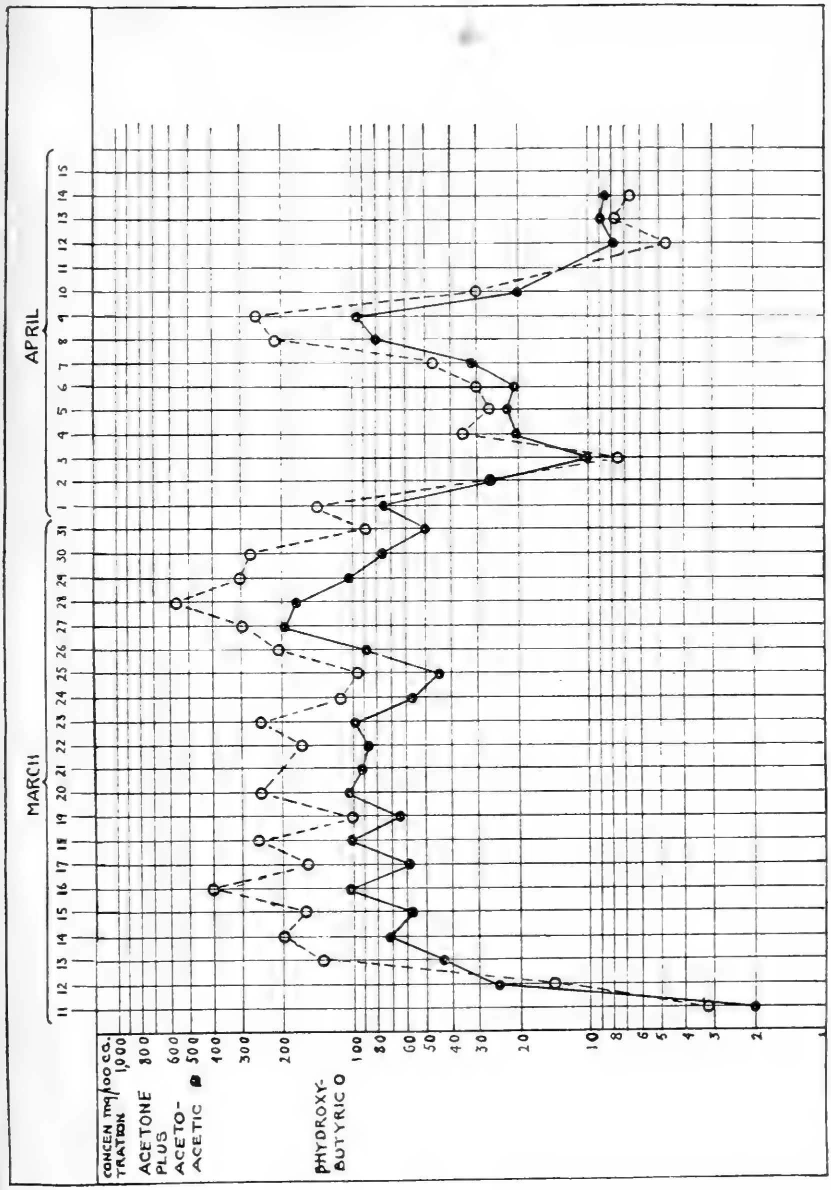

MARCH
APRIL
CONCEN mg/100 cc. TRATION
ACETONE PLUS ACETO-ACETIC
β HYDROXY-BUTYRIC

CHART 4A.

the acetone bodies varied from ten to one hundred times the values of those found when the subject was on a normal diet. It seems possible that the continued loss of weight and negative nitrogen balance noted in this case may be accompanied by a liberation of glycogen which would serve as an additional (endogenous) source of antiketogenic compounds, but as other experiments, as Case IV, do not show evidences of such a process during loss of weight this explanation can only be offered tentatively. The figures actually obtained show that the border-line diet was very close to the basal one, and as this is the only case in the series in which this was found to be true, it seems reasonable that some such phenomena as those suggested above may have diminished the excretion. The results show also that the ratio between the fat and carbohydrate rather than the amount of fat ingested determines the acetone excretion, for less acetone was found when the diet contained 153 gm. of fat and 45 gm. of carbohydrate than when it contained 133 gm. of fat and 36 gm. of carbohydrate. The excretion of the acetone bodies was so low that the determination of creatinine was not interfered with, and the determinations of this compound showed a high degree of success in collecting accurate 24 hour urines.

Case III, Mrs. M. H., was a woman 28 years old, 5 ft. 3 in. tall, and weighing 70 lbs. Her basal metabolism measured 1,240 calories per day. She had been suffering from severe chronic arthritis for 2 years, and was practically helpless. A special nurse was assigned to the case, and both the nurse and the patient cooperated well in carrying out directions. Altogether the results of the study of this case were very satisfactory, but in some instances specimens of urine were unavoidably lost due to the condition of the patient; the days on which these losses occurred are marked in the table with an interrogation point. The patient ate the entire amount of the diet provided at all times, and the diet furnished maintained the body weight throughout the experiment. It was possible to continue the base line diet long enough to determine the excretion of acetone caused by it with more accuracy in this case than in any other. Diets which had a lower ratio of carbohydrate to fat than did the basal diet caused a formation of larger amounts of acetone, and the change from one level of excretion to the other was gradual and not abrupt.

A diet which had a ketogenic power of 108 per cent caused practically no increase in the excretion of acetone; one having a value of 78 per cent caused an excretion of distinctly increased amounts, although these amounts were not great; while the basal diet—which has a value of 55 per cent—caused an excretion of between 1 and 2 gm. From these figures it would seem that the value of the border-line diet must lie at about 78 per cent, unless considerable importance is attached to the formation of very slight traces of the acetone bodies.

In this case small amounts of sodium bicarbonate were fed over a period of a few days after acetone excretion was thought to have reached an equilibrium which corresponded to the basal (10, 10, 80 per cent) diet. The sodium bicarbonate lowered the excretion of ammonia and of titra-

TABLE V.

Case V.

Date.	Diet. Protein.		Diet. Fat.		Diet. Carbohydrate.		Diet. Calories.	Weight.	Alveolar CO_2.	Urine. Volume.	Urine. Total N.	Urine. NH_4N	Urine. Acetone + diacetic.		Urine. β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*mm.*	*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
July 30	?	?	?	?	?	?	?			690		0.314	0.8	0.005	2.9	0.020
" 31	45	8.9	184	82.2	45	8.9	2,016	57.3	43	770	5.05	0.306	9.2	0.071	12.6	0.098
Aug. 1	46	9.1	181	81.8	45	9.1	1,989			820	8.10	0.586	48.2	0.394	89.7	0.734
" 2	45	9.5	170	81	45	9.5	1,890	57.0		830	7.20	0.518	83.0	0.688	153	1.27
" 3	45	10.5	149	79	45	10.5	1,701			800	5.70	0.488	92.0	0.735	178	1.42
" 4	45	9.6	167	80.8	45	9.6	1,879	56.2		770	6.70	0.736	120	0.924	307	2.46
" 5	45	8.9	174	77.3	72	13.8	2,036		36	500	5.00	0.500	129	0.645	458	2.29
" 6	45	8.8	176	77.5	72	13.7	2,052	56.4		620			122	0.754	371	2.30
" 7	45	9.0	170	77	72	14	1,998		36	440	3.86	0.478	80.6	0.360	410	1.80
" 8	45	8.7	178	77.8	72	13.5	2,070	56.5		540	5.00	0.660	86.6	0.467	188	0.865
" 9	45	8.9	153	69.1	117	23	2,025			570	3.00	0.324	40.0	0.188	51.8	0.296
" 10	45	8.0	178	71.2	117	20.8	2,250		38	180	1.50	0.150	44.8	0.081	41.1	0.074
" 11	45	8.0	178	71.2	117	20.8	2,250	57.4		700	2.90	0.188	55.0	0.035	7.8	0.055
" 12	45	10	150	64	67	26	1,800	56.8		260	1.49	0.100	38.3	0.100	29.4	0.076
" 13	35	9.6	121	59.1	67	31.3	1,497		34							
" 14	45	10.3	149	61.9	67	27.8	1,746									
" 15	45	10.3	150	63	67	26	1,800	57.0		500			46.7	0.234	38.3	0.191
" 16	47	10.2	152	64.2	69	25.6	1,832		34	390		0.108	25.0	0.098	28.4	0.111
" 17	46	10.2	149	63.7	68	26.1	1,797	57.3		570	4.06	0.365	25.8	0.147	31.4	0.179
" 18	45	10	150	63.9	68	26.1	1,800	57.2		340	2.72	0.243	81.5	0.277	165	0.563
" 19	45	10	150	63.9	68	26.1	1,800		41	410		0.293	60.0	0.246	109	0.563
" 20	46	10.2	138	62.2	67	27.6	1,699			530			49.2	0.261	89.4	0.411
" 21	?	?	?	?	?	?	?	57.6								

Results of determinations on the acetone bodies are expressed in terms of acetone.

table acid, diminished the degree of acidity of the urine, and increased the tension of carbon dioxide in the alveolar air. The amount of the acetone bodies and their concentration in the urine were increased while the subject received the drug, and returned to the level previously established after it was discontinued. The periods before and after the alkali was given were short, but the changes were so marked that the experiment probably indicates a real increase in the excretion of these compounds. The results are similar to those recorded by Joslin[4] and by Forssner (1911). As this was the only case in the series in which the effect of the administration of

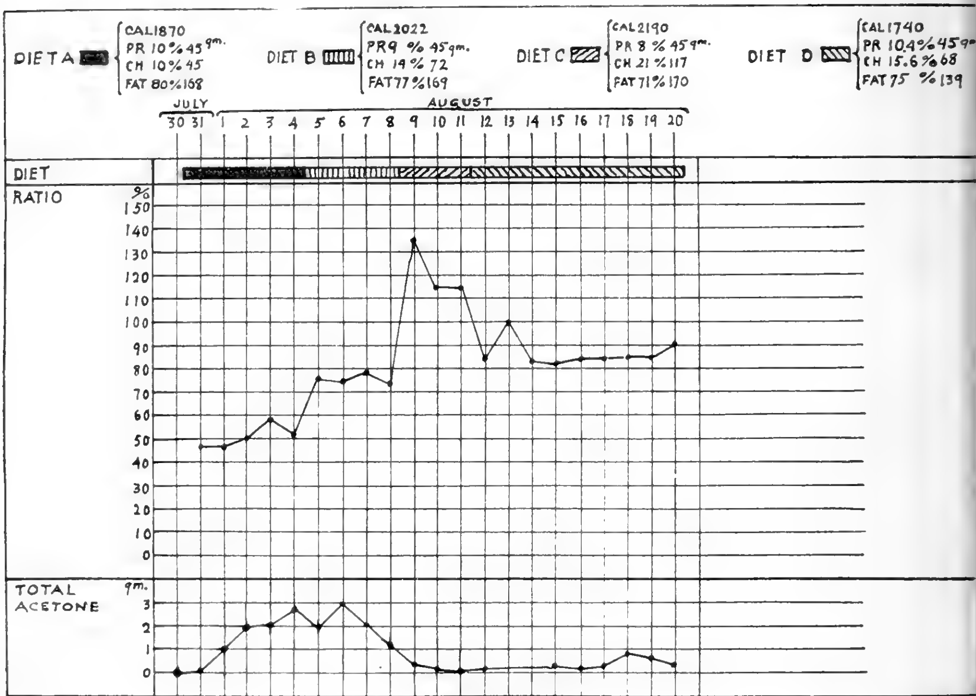


CHART 5.

alkali was studied, it is impossible to do more than note what may be an accidental finding.

Case V, Miss M. G., was a woman 22 years old, 5 ft. 2½ in. in height, who weighed 127 lbs. Her basal metabolism measured 1,430 calories per day. This experiment was much less satisfactory than those described above; the patient failed to eat all of the food provided in any of the diets, and also, apparently, to collect 24 hour urines in a satisfactory manner.

[4] Joslin (1917), pp. 394 and 395.

TABLE VI.

Case VI.

Date.	Diet.							Weight.	Urine.						
	Protein.		Fat.		Carbohydrate.		Calories.		Volume.	Total N.	NH_4 N	Acetone + diacetic.		β-hydroxybutyric acid.	
1921	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*		*kg.*	*cc.*	*gm.*	*gm.*	*mg./100 cc.*	*gm.*	*mg./100 cc.*	*gm.*
July 12	50	16	90	64.8	60	19.2	1,250		1,240			42.0	0.522	11.6	0.144
" 13	50	16	90	64.8	60	19.2	1,250	57.4	890		0.371	22.2	0.197	70.7	0.630
" 14	45	14.7	90	66.4	58	18.9	1,222								
" 15	45	14.7	90	66.4	58	18.9	1,222		870			23.3	0.203	19.7	0.171
" 16	45	14.7	90	66.4	58	18.9	1,222		1,055					19.7	0.207
" 17	46	15.6	86	65.7	55	18.7	1,178		1,000			25.0	0.250	25.5	0.255
" 18	47	16.1	84	64.7	56	19.2	1,168	57.5							
" 19	35	13.4	76	65.5	55	21.1	1,044		1,200	10.5	0.350	15.8	0.190	13.7	0.165
" 20	49	16.8	82	63.1	59	20.1	1,170		1,220			17.5	0.214	23.6	0.288
" 21	50	16	90	64.8	60	19.2	1,250		1,590			18.3	0.292	25.4	0.401
" 22	47	14.2	86	65.6	60	20.2	1,182		1,545			21.7	0.336	42.2	0.654
" 23	50	16	90	64.8	60	19.2	1,250		1,000			15.8	0.158	24.6	0.246
" 24	50	16	90	64.8	60	19.2	1,250		1,800			20.8	0.374	32.3	0.583
" 25	50	16.1	90	65.3	58	18.6	1,242		1,060			56.3	0.602	23.4	0.248
" 26	40	12.8	90	64.8	70	22.4	1,250	57.3	720			52.2	0.376	47.7	0.344
" 27	40	12.8	90	64.8	70	22.4	1,250		820			39.8	0.326	53.7	0.440
" 28	41	13.1	90	64.6	70	22.3	1,254		680			41.6	0.282	15.6	0.106
" 29	40	12.9	90	65	69	22.1	1,246		1,240		0.516	13.3	0.165	11.7	0.145
" 30	40	12.8	90	64.8	70	22.4	1,250		1,360			14.1	0.192	26.5	0.358

Results of determinations of the acetone bodies are expressed in terms of acetone.

Although the weight varied somewhat from day to day it was fairly well sustained throughout the period of study. It seems probable in such a case that fat not taken in the diet is replaced in metabolism by tissue fat, although the possibility of an increased combustion of glycogen must also be considered. This case showed acetonuria when the diet had a ketogenic balance of about 80 per cent, and the acetonuria practically cleared up when a diet having a balance of 110 per cent was fed.

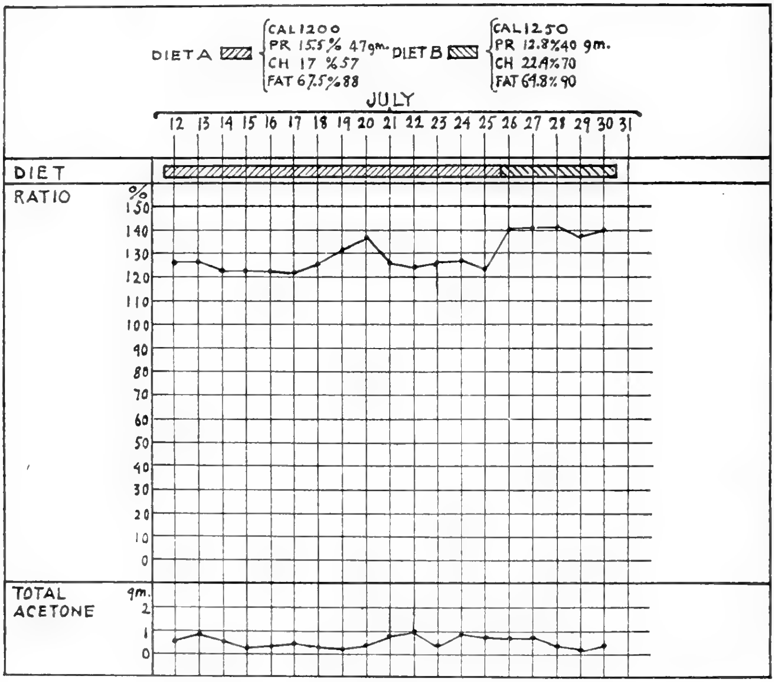


Chart 6.

Case VI, Mrs. E. Y., was a woman 70 years old, 5 ft. 2¼ in. tall, who weighed 135 lbs. Her basal metabolism measured 1,185 calories per day. There was a special nurse assigned to the case, but there was a lack of cooperation because the patient had a prejudice against protein food, and disliked large quantities of fat. This case was even less satisfactory than the preceding one. When the series of experiments was commenced the patient had been living on a diet low in carbohydrate for some time as a part of treatment for chronic arthritis. Acetone was found in her urine by the qualitative test used (Legal's) when the diets did not seem to be severe enough to cause the presence of the compound, and it seemed desira-

ble to determine whether there was an increased elimination of both the acetone bodies. There is no doubt that the patient showed such an increase, although no strictly normal values are available for comparison. The diet taken during the period of study contained, on the average 15 per cent of the calories as protein, 17 per cent as carbohydrate, and 68 per cent as fat, and had a ketogenic balance of 120 to 130 per cent. The results are different from those found on other subjects, and may perhaps be attributed to changes in the metabolism of the patient caused by her advanced age.

There is one fact which is evident in all of the experiments; when the diet was changed the level of the acetone excretion changed to correspond, but changed gradually. Why these changes should have taken 3 or 4 days in some instances cannot be explained in an entirely satisfactory way. One factor which delayed the response was undoubtedly the time which it took ingested fat to pass through the digestive, assimilative, and metabolic processes, but this did not seem adequate to account for the delay completely. It is possible that when there was a large excess of ketogenic compounds included in the diet, glycogen or other antiketogenic materials were furnished from the reserve supplies of the body in larger amounts than normal. If this was so not only would the changes be gradual, as was found to be the case, but also the amounts of acetone found during the period for which a given diet was fed would be lower than that expected from a calculation of the ketogenic balance of the diet. The data reported above are not sufficient to decide this question. Whatever may have been the cause of this gradual change in the acetone excretion, there are three facts which result from it: first, no decision concerning the acetone excretion which corresponds with a diet can be made until the diet has been fed for several days; second, analyses of the fat content of stools is not necessary because it would not be possible to decide to what acetone excretion the figures would apply; third, it is useless to feed the diets—at least to feed the fat content of the diets—in small amounts taken frequently.

In interpreting the meaning of the excretion of acetone when diets are fed, which are at or near the border-line of ketogenic antiketogenic equilibrium, there are certain possibilities which must be kept in mind. For instance, the body tissues may furnish part of the material burned, and this will be of ketogenic or

antiketogenic nature, as it may be derived from fat, protein, or glycogen. It is probable that a mixture of these is consumed in amounts which will appreciably affect the excretion of acetone when the subject is losing weight, and may so affect it at other times. The composition of the different materials burned may vary with the general nutrition of the patient, and with other factors not understood, factors which are perhaps similar to those which affect the storage of fat.

Another fact which must be taken into consideration, especially when slight increases of acetone excretion are studied, is the probable variation in the mixtures of foodstuffs burned in the body at different times during the 24 hour periods. In the study of the effect of diets on the excretion of the acetone bodies discussed here the interpretation has been based on the analysis of 24 hour specimens of urine; the results of these analyses have been discussed as if they represented not only the total, but also the average excretion for the periods. Such an assumption is not correct, because when the ketogenic and antiketogenic compounds are present in equivalent amounts, or when the antiketogenic material is present in excess, no matter how great that excess may be, no acetone bodies will be formed, while if the ketogenic material is in excess they will be formed.

Although the nature of the results showed that it could not make much difference how the fat in any given diet was fed, it did seem possible, because carbohydrate is more rapidly assimilated, that feeding small amounts of this foodstuff frequently would reduce the error described above. In one of the cases in the series the carbohydrate was given at six times during the day, but practically the same amount of acetone was found as when it was given in three meals.

It is possible that different combinations of foodstuffs may be simultaneously oxidized in different parts of the organism. If the cells were burning mixtures of foodstuffs which were at or near the border-line of ketogenic antiketogenic equilibrium, it is conceivable that slight disturbances of the blood and nutriment supplied to various parts of the body would lead to a local production of acetone. A similar explanation has been suggested to account for the rise in blood acetone found after the injection of small amounts of adrenalin chloride (Hubbard and Wright,

1921). It does not seem probable that this effect can be of as much importance in producing an excretion of acetone as can differences in metabolism at different times during the day, but the possibility that there is some such source of acetone cannot be neglected. If the age of the patient studied in the sixth experiment has any effect upon the formation of the acetone bodies it was probably to exaggerate the effect either of the "local" or "temporary" production of those compounds.

The various uncertain factors which must influence the interpretation of the results of these experiments may be summarized as affecting three different parts of the study: one, as influencing the value used to express the ketogenic balance of the diets; two, as rendering uncertain the accuracy with which the excretion of the acetone bodies observed corresponds to that which should have been found; and, three the interpretation of the results in terms of ketogenic equilibrium. Uncertainties which affect the value of the ratio include: one, the use of a figure to convert the sum of the antiketogenic factors into terms of fatty acid which is based on the molecular weights of the higher fatty acids; two, the use of the total carbohydrate content of the diet instead of its glucose equivalent in calculating the antiketogenic compounds; three, the use of the fat fed instead of the fat absorbed for measuring the amount of the ketogenic compounds; and, four, the uncertainty as to the correct percentage of protein which figures as a source of antiketogenic material. Of these uncertainties the first is the only one which would certainly increase the apparent ketogenic value of the expression, the second and third sources of error would certainly increase its antiketogenic value, while the effect of the fourth is undeterminable. The errors which may have affected the experiments themselves are of various kinds: first, the subjects did not always eat all of the food which was provided; second, they may have eaten articles of food which were not provided; third, enough protein was not eaten in all cases exactly to maintain the subjects in nitrogen equilibrium; fourth, enough food was not taken in all instances to maintain metabolic equilibrium; fifth, 24 hour specimens of urine were not always accurately collected. The first three sources of error mentioned were such as would lead to real or apparent increases in the amount of antiketogenic compounds metabolized,

as fat was the food which was left untouched, carbohydrate was the food which the subjects most craved, and a negative nitrogen balance was observed more frequently than was a positive one; the effect of the loss in weight and of the failure to collect urines accurately cannot be determined. A detailed examination of these sources of error has shown that either they could not be wholly avoided, or that their influence on the results was slight.

The difficulties which affect the interpretation of the excretion of the acetone bodies influence the study of the results of the experiments more than do the other uncertainties met with in these experiments. These sources of uncertainty include: temporary production of the acetone bodies due to variations in the foodstuffs burned at different times during the day; local production of the acetone bodies caused by variations in different parts of the organism; and, possibly, the effect of glycogen drawn from the reserve stores of the body. All of these except the last would lead to a production of acetone greater than the composition of the diets would indicate.

In interpreting the value of the expression

$$100 \times \frac{1.5 \text{ (weight carbohydrate} + 25 \text{ per cent weight protein)}}{95 \text{ per cent weight fat}} = \text{N per cent}$$

which will express the condition of ketogenic antiketogenic equilibrium, the effect of these uncertainties, particularly of those affecting the interpretation of small amounts of the acetone bodies, must be kept in mind. When the value of the expression was 100 per cent or more, acetone was not found in the urine except in very small amounts, and in two of the cases studied, the excretion decreased when the diets had this value to the normal level. In one other case such a diet failed to cause the appearance of a distinctly increased acetonuria, although the period of study (3 days) was perhaps not long enough to produce an equilibrium in the body. When diets were fed which gave numerical values between 55 and 60 per cent rather large amounts of acetone were excreted; there was a distinctly increased excretion also, except in Case II, when diets giving values of about 80 per cent were taken. It seems most reasonable to attribute the small amounts of acetone found on the diets which figured at 100 per cent to local and temporary production of the acetone bodies, and to conclude that values of

80 to 90 per cent approximately represent the diet in which the ketogenic and antiketogenic foods are present in equivalent amounts.

It has been shown already that certain numerical values of the expression when ketogenic equilibrium is attained correspond to the different possible antiketogenic effects of the glycerol radicle: if glycerol does not figure as a source of antiketogenic compounds the value is 100 per cent; if glycerol is converted into glucose, and this glucose takes part in the reaction between ketogenic and antiketogenic compounds, the value is 83 per cent; if glycerol takes part in the reaction as a three carbon atom residue, the value is 67 per cent. A comparison of these values with the one which has been found experimentally to correspond with the condition of equilibrium makes it seem most probable that the glycerol residue of the fats does figure only to the extent to which it can yield glucose. These conclusions would be expressed mathematically as follows:

$$100 \times \frac{1.5\ (\text{weight carbohydrate} + 25\ \text{per cent weight protein})}{95\ \text{per cent weight fat}} = 83\ \text{per cent}$$

If this equation is transposed so as to express the amounts of protein, fat, and carbohydrate which should be fed to produce a condition of ketogenic equilibrium, the expression becomes:

$$1.9\ (\text{weight carbohydrate} + 25\ \text{per cent weight protein}) = \text{fat}.$$

This expression is practically identical with that stated by Woodyatt (1921):[5] "2 × carbohydrate + protein = fat."

It is of course possible that too much stress has been laid upon "temporary" and "local" sources of traces of acetone, and that not enough emphasis has been placed upon glycogen as a source of antiketogenic compounds. If very small amounts of acetone result from an excess of antiketogenic material in the diet, 100 per cent probably represents the condition of equilibrium. In this case the expression would be:

$$100 \times \frac{1.5\ (\text{weight carbohydrate} + 25\ \text{per cent weight protein})}{95\ \text{per cent weight fat}} = 100\ \text{per cent}$$

and the expression for the relative amounts of food would become:

[5] Woodyatt (1921), p. 133.

$$1.42 \text{ (weight carbohydrate} + 25 \text{ per cent weight protein)} = \text{weight fat.}$$

This expression probably does not express the condition of ketogenic equilibrium correctly; the one given above is almost certainly preferable.

It seems reasonable to conclude from the experiments reported that the two and three carbon atom residues from the α-amino-acids do not figure directly in the antiketogenic reaction, but are condensed to glucose. If these residues did react with the ketogenic compounds the numerical value for each diet would be higher than they are reckoned here; acetonuria would develop and clear up at values of from 100 to 120 per cent, and traces of acetone would be found in some cases when the value was 150 per cent.

The charts and tables recording these experiments have been examined for evidence of an adaptation of the organism to these diets high in fat with a consequent reduction of the amounts of the acetone bodies excreted. Folin and Denis (1915) have reported evidences of such an adaptation to starvation in three obese women studied by them, but there did not seem to be such a response to diets high in fat. When the basal diet—containing 10 per cent of the calories in the form of protein, 10 per cent in the form of carbohydrate, and the balance in the form of fat—was resumed after periods during which diets containing relatively more or less fat was fed, the excretion of acetone returned to the level first established if the base line diet was continued over a sufficient period.

The method adopted of plotting the concentration of the acetone bodies upon paper ruled with logarithmic characteristics shows clearly the relationship between the two fractions of the acetone bodies discussed in an earlier paper (Hubbard, 1921). When large amounts of the acetone bodies were excreted the acetone from β-hydroxybutyric acid was in excess of that from preformed acetone plus acetoacetic acid, but when the concentrations were only slightly increased the two fractions were as a rule nearly equal; in some cases the acetone from preformed acetone plus acetoacetic acid was in excess. When acetonuria developed slowly it was this fraction which increased first, while the β-hydroxybutyric acid increased later. The interpretation of these facts is complicated by differences in the kidney thresholds of the different acetone bodies.

Other results which have not yet been discussed include changes in alveolar carbon dioxide tension, the excretion of ammonia and of titratable acid, and changes in the reaction of the urine. The urinary ammonia roughly paralleled the acetone bodies except when sodium bicarbonate was added to the diet; during that period the excretion of ammonia was markedly reduced, while that of the acetone bodies was somewhat increased. The alveolar carbon dioxide tension was somewhat lowered by the more extreme diets, and the values returned to normal when sodium bicarbonate was taken. The variations of the titratable acidity and hydrogen ion concentration were little greater than those which are normally found; these were, of course, markedly affected by the administration of the alkali.

CONCLUSION.

A method has been suggested for expressing the ketogenic ballance of any diet mathematically. A series of six experiments has been described in which the effect of diets high in fat on the excretion of the acetone bodies by normal subjects was studied, and the results compared with this mathematical expression. From the results obtained the following conclusions have been drawn: (1) that the mechanism which controls the formation of increased amounts of the acetone bodies can be regarded as a molecular reaction or balance between ketogenic substances such as the fatty acids and antiketogenic substances such as glucose; (2) that protein figures as an antiketogenic compound only to the extent of the glucose which it can yield in the organism; (3) that glycerol, when fed as a part of the fat molecule figures as an antiketogenic compound only to the extent to which it forms glucose in the organism; and (4) probably that glycerol so fed does figure as an antiketogenic compound to the extent to which glycerol itself can yield glucose.

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THE RESOLUTION OF HYDROXYASPARTIC ACIDS INTO OPTICALLY ACTIVE FORMS.

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In a recent communication the synthesis and separation of two inactive forms of hydroxyaspartic acid have been described (1). The inactive form, more soluble in water, gave mesotartaric acid on treatment with nitrous acid and was designated as the *anti* compound, while the less soluble form gave racemic acid under similar conditions and was named the *para* compound. Each of these inactive acids contains two dissimilar asymmetric carbon atoms and should be resolvable into active components, giving a total of four active and two inactive forms. The resolution of the *anti* acid was readily effected by means of alkaloids as described in the present communication, but the *para* acid could not be resolved by this method although its finely crystalline alkaloid salts were subjected to exhaustive fractional crystallization. It appears that the alkaloid salts of the *para* acid are partially racemic compounds of the type described by Ladenburg (2). On turning to alternative biological methods for the resolution of the *para* acid it was found that no resolution could be effected by growing *Penicillium glaucum* in solutions of the sodium salt while some rather inconclusive evidence was secured of a slight resolution by fermenting yeast used according to Ehrlich's method (3). The small amount of dextro-rotatory acid thus obtained gave dextro-tartaric acid on treatment with nitrous acid. Since the Walden inversion rarely occurs with nitrous acid it is probable that *d*-hydroxyaspartic acid and *d*-tartaric acids are similarly constituted, and the same would be true of the *levo* forms. Both active forms of *anti*-hydroxyaspartic acid give inactive mesotartaric acid on treatment with nitrous acid so that their relative configuration remains undecided. On heating either of the active *anti* acids with water at 125° partial conversion into the

para acid was effected, but the latter was invariably optically inactive.

It is perhaps somewhat surprising that the active *anti*-hydroxyaspartic acids should have as high a specific rotation as 12°. In the light of various theories of optical superposition it might be anticipated that a substance derived from internally compensated mesotartaric acid by the replacement of one hydroxyl group with a relative mass of 17 by an almost equally heavy amino group with a mass of 16, would have a vanishingly small rotation but this is evidently not the case. Of course the possibility still exists of a Walden inversion taking place in the action of nitrous acid on hydroxyaspartic acid, but at present the evidence is against such an assumption.

Resolution of Inactive Anti-Hydroxyaspartic Acid.

The inactive *anti* acid, prepared as previously described, gives well crystallized salts with quinine, brucine, and strychnine and resolution may be effected by fractional crystallization of any of them. On the whole the most satisfactory plan is to separate the dextro acid first as strychnine salt and to use quinine for the separation of the levo acid. The morphine, cinchonine, and quinidine salts were not found helpful for purposes of resolution.

Strychnine d-Anti-Hydroxyaspartate.—10 gm. of *anti*-hydroxyaspartic acid were heated on a water bath with 75 cc. of water and slightly less than the theoretical amount of strychnine was added by degrees. The clear solution was then set aside to crystallize in an ice box. The salt crystallized readily in laminated plates which were filtered off and washed with 50 per cent aqueous acetone. The yield of crude strychnine salt is approximately the theoretical amount calculated for the dextro component and was obtained apparently optically pure after two further crystallizations from water (50 cc.). The air-dried salt contains close to 4 molecules of water which were removed on drying at 120° over phosphorus pentoxide under greatly reduced pressure. The salt is insoluble in acetone and moderately soluble in methyl and ethyl alcohol. It crystallizes best from water in which it is very soluble when hot, but sparingly soluble at low temperature.[1]

[1] The optical rotations recorded in this paper were observed in a highly sensitive Schmidt and Haensch polarimeter, for the use of which instrument I am indebted to The Rockefeller Institute for Medical Research.

0.1430 gm. air-dried salt lost 0.0183 gm. H_2O at 120° = 12.9 per cent.
Calculated for $C_{21}H_{22}N_2O_2 \cdot C_4H_7O_5N \cdot 4H_2O$ = 13.0 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.2; $\alpha = -0.42°$
$[\alpha]_D^{20°} = -19.1°$

d-Anti-Hydroxyaspartic Acid.—The strychnine salt obtained as just described was dissolved in hot water and the bulk of the strychnine precipitated with a slight excess of ammonia. The filtrate was then extracted repeatedly with a mixture of amyl alcohol and ether until the remaining trace of strychnine was removed. The solution was then concentrated under diminished pressure to a small bulk (15 cc.) and rendered just acid to Congo red by the cautious addition of dilute nitric acid. On allowing the solution to stand in a cool place the dextro acid separates out readily in the form of transparent thick wedge-shaped prisms which only become opaque after long standing. For analysis the acid was dried over phosphorus pentoxide at 60°.

0.1656 gm. substance : 0.1971 gm. CO_2, 0.0724 gm. H_2O.
$C_4H_7O_5N$. Calculated. C 32.2, H 4.7.
Found. " 32.5, " 4.8.
Rotation. c = 2.0 in water; l = 2.2; $\alpha = +0.53°$
$[\alpha]_D^{20°} = +12.1°$

The dextro acid is slightly less soluble in water than the inactive acid, dissolving in about 45 parts of water at room temperature compared with about 30 parts for the latter. The chemical properties of the active acid as expected closely resemble those of the inactive acid. An interesting fact was noted that on recrystallizing a mixture of the dextro and inactive acids from water, the mother liquor which was at first dextro-rotatory, on standing in contact with the separated crystals gradually became entirely inactive while the separated dextro acid increased in amount. It is inferred that the inactive acid is a *dl*-mixture at any rate in solution at room temperature and not truly racemic, since under these circumstances the shifting of the equilibrium is readily comprehensible. The yield of pure dextro acid from 10 gm. of the inactive compound was 3.2 gm. A slightly larger yield may be obtained by separating the acid as lead salt rather than by direct crystallization, but the optical purity of the product is apt to be impaired. The optical rotation of the dextro acid is increased about 30 per cent on addition of hydrochloric acid. Most of the soluble salts are also dextro-rotatory.

Quinine l-Anti-Hydroxyaspartate.—The mother liquor from the strychnine salt of the dextro acid may be utilized conveniently for the preparation of this salt although it may also be obtained direct from the inactive acid. The strychnine mother liquors are precipitated with ammonia, and a crude levo acid obtained by neutralizing the concentrated filtrate with nitric acid using Congo red as indicator. The acid crystallizes readily on keeping in a cool place.

The crude levo acid (6 gm.) was suspended in 75 cc. of water and quinine base (15 gm.) added by degrees while heating on the water bath. The clear solution on cooling quickly deposited long needles of the salt which were filtered off on the following day. The yield of air-dried salt was 12.7 gm. and its specific rotation was −96.4. On recrystallization from water (20 cc.) the rotation was practically constant at −95.5. The salt contains close to 4 molecules of water of crystallization.

0.1830 gm. air-dried salt lost 0.0238 gm. H_2O at 110° = 12.9 per cent.
Calculated for $C_{20}H_{24}N_2O_2 \cdot C_4H_7O_5N \cdot 4H_2O$ = 13.2 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.2; $\alpha = -2.10°$

$$[\alpha]_D^{20°} = -95.5°$$

l-Anti-Hydroxyaspartic Acid.—The quinine salt above described was dissolved in hot water and most of the quinine precipitated by a slight excess of ammonia. The filtrate was repeatedly extracted with chloroform to remove the remaining alkaloid and then concentrated under diminished pressure to about 15 cc. Dilute nitric acid was then added until the reaction was just acid to Congo red and the free acid crystallized readily. A single recrystallization from hot water gave an optically pure product. The acid crystallizes in wedge-shaped prisms, soluble in about 45 parts of water at room temperature and save for its sign of rotation it has properties identical with those of the dextro acid. For analysis it was dried at 60° over phosphorus pentoxide.

0.1747 gm. substance : 0.2051 gm. CO_2, 0.0781 gm. H_2O.
$C_4H_7O_5N$. Calculated. C 32.2, H 4.70.
Found. " 32.0, " 4.95.
Rotation. c = 2.0 in water; l = 2.2; $\alpha = -0.52°$

$$[\alpha]_D^{20°} = -11.9°$$

Action of Nitrous Acid on d- and l- Anti-Hydroxyaspartic Acids.—In each case 0.5 gm. of the active acid was dissolved in 20 cc. of water together with 0.5 cc. of concentrated hydrochloric acid. Silver nitrite (0.7 gm.) was added by degrees in the course of 24 hours. The mixture was allowed to stand for a further day after which the reaction was complete. Silver chloride was then filtered off and the filtrate concentrated to 15 cc. On examination in a 2.2 dm. tube the solutions were found to be absolutely inactive and on addition of ammonia and calcium acetate a large yield of the characteristic calcium mesotartrate was at once obtained. It was evident that no trace of the active tartaric acids had been produced.

Experiments on the Resolution of Inactive Para-Hydroxyaspartic Acid.

Strychnine Para-Hydroxyaspartate.—3 gm. of the *para* acid suspended in 25 to 30 cc. of water were heated on the water bath with the gradual addition of 6.6 gm. of strychnine. The salt crystallized very readily in regular prisms and was filtered off and recrystallized twice more from 20 cc. of water. The air-dried salt contained 3 molecules of water of crystallization and its specific rotation was constant at $-23.2°$. The yield of thrice crystallized salt was close to 50 per cent.

0.1404 gm. lost 0.0140 gm. H_2O at 110° = 10.0 per cent.
Calculated for $C_{21}H_{22}N_2O_2 \cdot C_4H_7O_5N \cdot 3H_2O$ = 10.1 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.2; $\alpha = -0.51°$

$$[\alpha]_D^{20°} = -23.2°$$

On decomposing the strychnine salt as already described for the *anti* compound, a good yield of the *para* acid was recovered. It was absolutely inactive in a saturated solution, as was also the mother liquor from which it had crystallized. On treating the mother liquors from the strychnine salt in the same way, the inactive acid was again recovered while the mother liquor which still contained a minute trace of strychnine had an angular rotation of only $-0.04°$ in a 2.2 dm. tube. From these results it is clear that no resolution had been accomplished by the fractional crystallization of the strychnine salt.

Cinchonine Para-Hydroxyaspartate.—The *para* acid (2.5 gm.) and cinchonine (5 gm.) were dissolved in 30 cc. of water. A separation of exceptionally fine clear hard hexagonal prisms readily occurred on cooling. The first crop of crystals (6.0 gm.) was recrystallized twice more from water (20 cc.). The air-dried salt, representing about 45 per cent of the theoretical amount, contains rather more than 2 molecules of water of crystallization.

0.1152 gm. lost 0.0101 gm. H_2O = 8.77 per cent.
Calculated for $C_{19}H_{22}N_2O \cdot C_4H_7O_5N \cdot 2H_2O$ = 7.66 " "
Rotation. c = 1.0 air-dried salt in water; l = 2.0; $\alpha = +2.45°$

$$[\alpha]_D^{20°} = +122.5°$$

On decomposing the cinchonine salt as already described, an absolutely inactive *para*-hydroxyaspartic acid was recovered. The mother liquors from the cinchonine salt on similar treatment gave exclusively the inactive acid showing that no resolution had been accomplished.

Brucine Para-Hydroxyaspartate.—This salt which is extremely soluble in water was crystallized from alcohol. The acid (2.5 gm.) was neutralized with brucine (7.5 gm.) in hot aqueous solution which was then evaporated to a syrup and stirred with about 4 volumes of absolute alcohol. The salt separates out readily in fine thin plates containing 4 molecules of water of crystallization. The anhydrous salt is extremely hygroscopic and was dried over phosphorus pentoxide at 110° under reduced pressure.

0.3000 gm. air-dried salt lost 0.0355 gm. H_2O = 11.8 per cent.
Calculated for $C_{23}H_{26}N_2O_4 \cdot C_4H_7O_5N \cdot 4H_2O$ = 11.7 " "
Rotation. c = 2.0 air-dried salt in water; l = 2.2; $\alpha = -1.03°$

$$[\alpha]_D^{20°} = -23.4°$$

The recrystallized brucine salt gave on decomposition only the inactive *para* acid and the same was recovered exclusively from the mother liquors, showing that no resolution had been effected.

Quinine Para-Hydroxyaspartate.—This salt readily crystallizes in masses of fine felted needles on warming 2.3 gm. of the acid with quinine (5.8 gm.) in 30 to 40 cc. of water. The salt was crystallized from water four times, but the acid recovered either from the salt or mother liquor was optically inactive. The air-dried salt retains 2 molecules of water of crystallization. The

anhydrous salt obtained by drying under reduced pressure at 120° is very hygroscopic.

0.1537 gm. air-dried salt lost 0.0108 gm. H_2O = 7.02 per cent.
Calculated for $C_{20}H_{24}NO_2 \cdot C_4H_7O_5N \cdot 2H_2O$ = 7.09 " "
Rotation. c= 1.0 air-dried salt in water; l = 2.2; $\alpha = -2.55°$

$$[\alpha]_D^{20°} = -116°$$

Action of Penicillium on Para-Hydroxyaspartic Acid.—A gram of the acid was converted into the monosodium salt by neutralizing with sodium hydroxide, using litmus as indicator. The solution was then diluted to 200 cc. with an inorganic nutrient solution and heavily sown with *Penicillium glaucum.* The solution contained in a beaker was loosely covered with a clock-glass and no special precautions were taken to exclude infection with other organisms, since it appears that in many cases a satisfactory resolution is more often accomplished with mixed cultures than with a single pure organism. An excellent growth of the mold was obtained and at the end of 3 weeks the solution was just acidified with acetic acid, boiled with a little charcoal, and filtered. The filtrate was entirely inactive and on recovering some of the unchanged acid by means of the lead salt, it also was found to be inactive. No resolution had been affected therefore.

Action of Fermenting Yeast on Para-Hydroxyaspartic Acid.—These experiments were made substantially in accord with Felix Ehrlich's (3) excellent method for the resolution of inactive amino-acids. 2 gm. of the *para* acid were converted into the monosodium salt and then mixed with a solution of cane-sugar (50 gm.) in water (500 cc.). 50 gm. of carefully washed yeast were then added and fermentation was allowed to proceed to completion which took 4 to 5 days at 28°. The filtered solution which contained no sugar was then concentrated under reduced pressure and the unchanged acid recovered by precipitation with lead acetate in neutral solution. The lead precipitate was filtered off, well washed, suspended in hot water, and decomposed with hydrogen sulfide. The bulk of the acid recovered, which was about 0.8 gm., was optically inactive and readily crystallized. The mother liquor, however, had a definite rotation of + 0.19° in a 2.2 dm. tube and apparently represented a weak solution (less than 1 per cent) of the dextro and inactive acids On treatment with hydrochloric

acid and silver nitrite the rotation of the solution was not abolished as was the case with the active *anti* acids but was slightly increased (+0.24°). On concentrating the solution and adding potassium acetate, a separation of acid potassium tartrate was easily obtained. On dissolving the washed crystalline tartrate in dilute hydrochloric acid, a distinct dextro-rotation (+0.11°) was observed in a 2 dm. tube and this rotation was increased on addition of Walden's uranium reagent. The results of this experiment, which were confirmed by repetition, made it appear probable that a small amount of resolution of the inactive *para*-hydroxyaspartic acid had been affected and that the levo component was preferentially utilized by the yeast. Furthermore, it appears that the *d*-amino-acid gives *d*-tartaric acid on treatment with nitrous acid. The amount of resolution effected by yeast was insufficient to hold out much hope of isolating the pure active acid by its action.

Addendum.

The Probable Absence of the Hydroxyaspartic Acids in Casein.—The isolation of the two inactive and some of the active forms of hydroxyaspartic acid referred to in the preceding paper made it possible to search more intelligently for this amino-acid among the products of protein hydrolysis. A careful examination of the products from casein has failed to reveal its presence and furnishes additional evidence for the rejection of Skraup's statements to the contrary.[2]

Owing to the relative instability of hydroxyaspartic acid to the prolonged action of acids, the casein was hydrolyzed by tryptic digestion over 5 months. The neutral monoamino-acids were removed by extraction with butyl alcohol and the residue was then precipitated with lead acetate in neutral solution. The lead precipitate was decomposed with sulfuric acid and the filtrate warmed with excess calcium carbonate in order to remove most of the phosphates. The acid calcium salts of hydroxyaspartic acid are fairly soluble and should be formed in the filtrate if present. The filtrate was concentrated to about 200 cc. and the excess of barium hydroxide added with a view to obtaining the insoluble neutral barium salts of hydroxyaspartic acids. The

[2] Compare Dakin, H. D., *J. Biol. Chem.*, 1921, xlviii, 273.

precipitate on decomposition with sulfuric acid was found to contain a little organic phosphorus but very little (0.11 gm.) amino nitrogen. No hydroxyaspartic acid could be induced to crystallize even after "seeding," nor could its phenylisocyanate derivative be obtained. It is concluded that no significant amount of hydroxyaspartic acid is formed by the tryptic digestion of casein.

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THE HYDROGEN ION CONCENTRATION AND BICARBONATE LEVEL OF THE BLOOD IN PNEUMONIA.*

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Certain studies of the acid-base equilibrium in disease were reported from this clinic a year ago (1).

The method employed was in brief the determination of the carbon dioxide dissociation (or absorption) curve, and the carbon dioxide content of the patient's arterial and venous blood, and from these data the construction of the carbon dioxide diagram of Haggard and Henderson (2).

The method was described in the first paper (1). The further studies reported here were carried out in an identical manner, except that the Van Slyke (3) blood gas apparatus was used instead of the Henderson (4). The combined method of Van Slyke and Stadie (5) was used. In the present research all arterial or A-points, and all venous or V-points, were placed on the dissociation curve at its intersection with the abscissa representing the carbon dioxide content of the blood as found by analysis. All the curves obtained are shown in Figs. 1 to 7. They are all for blood equilibrated with air and various tensions of carbon dioxide at the patient's body temperature.

Since in venous blood and in some pathologic arterial bloods there is an oxygen unsaturation, the curve obtained for fully aerated blood does not truly represent that of the blood as it exists in artery or vein. The effect of oxygen unsaturation according to

* This paper is No. 26 of a series of papers on the physiology and pathology of the blood from the Harvard Medical School and allied hospitals, a part of the expense of which has been defrayed by a grant from the Proctor Fund for the study of chronic disease. A parallel and more extensive study of the acid-base balance in pneumonia has been carried out at the Boston City Hospital by Drs. Buckman, Adams, and Edwards. These results will be published shortly as a part of this series of papers.

Haldane and his coworkers (6) and to Peters, Barr, and Rule (7) is to shift the curve upwards. According to Haggard and Henderson (8) this phenomenon does not take place in oxalated blood. The blood used in this research was oxalated, but since Henderson and Haggard's findings have not been confirmed by others we have here plotted the A- and V-points on the curves for fully oxygenated blood but have also calculated the possible effect of oxygen unsaturation by the formula of Peters, Barr, and Rule (7). The position of the points when so corrected is shown in the several figures by circles containing crosses, the uncorrected points by plain circles.

As has been shown by Haggard and Henderson, the position of A- or V-points with respect to a series of radii drawn through the zero point gives the hydrogen ion concentration of the blood. In the figures in this paper the diagonals for various hydrogen ion concentrations (pH) have been constructed as in the paper of Peters, Barr, and Rule (7), and in Table I is given the pH for the various points, both corrected for oxygen unsaturation, and uncorrected, as read off from these diagonals. The diagonals so constructed, however, are for a temperature of 38°C. Since the solubility of carbon dioxide in blood is one of the factors used in the construction of the pH diagonal, and since the solubility varies with the temperature, the position of the diagonal will also vary with the temperature. The solubility coefficient of carbon dioxide in whole blood according to Bohr (9) is 0.937 at 15°C. and 760 mm., and is 0.511 at 38°C. and 760 mm. If the temperature solubility coefficient curve is a straight line (which may legitimately be assumed for the present purpose), then the solubility coefficient at 40°C. and 760 mm. would be 0.474. Using this coefficient for the construction of a given pH line, we find that what it amounts to is that at 40° a given diagonal represents a pH that is lower by 0.04 than that at 38°. This correction has been made in the table by adding 0.01 to the pH as read from the diagonals for each half degree of temperature elevation above 38°. In the figures it is shown by moving the A- and V-points in relation to 38° diagonals, this being more satisfactory than drawing complete sets of diagonals for each patient's temperature. The points so corrected are shown by black dots. These black dots then probably represent the true pH of the blood as

nearly as we can measure it by this method, since they are corrected both for oxygen unsaturation and for body temperature. The temperature correction is an approximation; it is probably maximal and sufficiently near the truth for the present purpose.

The studies presented in this paper deal entirely with the blood of pneumonia patients. In the previous paper (1) the diagrams for the bloods of three pneumonia patients were shown. We felt at that time that there was some evidence, from the position of the A-points, of an increase in the hydrogen ion concentration of the blood of these patients. Peters has criticized this conclusion on the basis that the effect of oxygen unsaturation was not taken into account and that, if it were, the hydrogen ion concentration of the three bloods under discussion would be found quite normal. Whether this is a valid criticism or not will depend on who proves correct in the controversy over the effect of oxygen unsaturation on oxalated blood. We will not enter this controversy now. The object of the present research was by further study to try to discover whether any change in hydrogen ion concentration or level of blood bicarbonate occurs in pneumonia. In our figures and table the pH, uncorrected, corrected for oxygen unsaturation, and corrected for both oxygen unsaturation and body temperature may all be found.

In this communication we will present for discussion seventeen carbon dioxide diagrams of the bloods of ten pneumonia patients. These patients were also made the subject of a study of the effect of oxygen therapy. Their histories have been reported in full in that connection by Barach and Woodwell (10), so that it will be unnecessary to repeat them here; the case numbers used here are the same as those used by Barach and Woodwell.

All data except the actual curves will be found in Table I, the curves themselves in Figs. 1 to 7. Ten of the seventeen curves were obtained during the height of the disease and before the patients had received any alkali or oxygen therapy. The remaining seven were taken either after crisis or after treatment.

The Blood pH in Pneumonia.

The matter of the state of the acid-base balance in pneumonia may for convenience be separated into the related matters of blood reaction as indicated by hydrogen ion concentration (pH)

Case No.*	Date.	Diagnosis.	Day of disease.	Temperature.	Oxygen saturation.		Carbon dioxide content.		Carbon dioxide tension.		pH uncorrected	
					A†	V†	A	V	A	V	A	
	1920			°C.	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*mm.*	*mm.*		
11	Nov. 22	Lobar pneumonia.	3rd	38.5	98.8	66.0	53.5	56.3	37.0	44.0	7.41	7
14	" 30	" "	7th	39.5	74.4	54.8	47.0	46.4	51.0	49.5	7.21	7
	Dec. 2		9th	38.5	91.6	36.0	47.2	53.7	36.0	71.5	7.36	7
22	" 11	Bronchopneumonia. Septicemia.	7th	40.2	91.9		37.8		40.5		7.21	
23	" 13	Lobar pneumonia.	4th	40.2	78.6	65.9	51.6	52.8	51.5	54.0	7.24	7
	1921											
17	Jan. 4	Bronchopneumonia. Septicemia.	21st	39.5	93.9	77.1	42.4	43.8	39.0	42.0	7.27	7
	" 5		22nd	39.0	97.7	85.2	44.5	46.5	24.5	29.0	7.51	7
13	" 5	Lobar pneumonia.	5th	40.3	92.4		42.0		41.5		7.25	
	" 18			36.4	96.5		50.6		45.0		7.29	
20	" 28	Lobar pneumonia.	7th	40.0	92.5	64.1	36.6	41.4	45.5	59.5	7.15	7
	Feb. 15			37.0	96.2		55.0		44.0		7.35	
10	Jan. 31	Lobar pneumonia. Pulmonary tuberculosis.	6th	39.1	77.2	50.2	49.5	53.1	46.0	53.0	7.27	7
	Feb. 2		8th	39.1	62.3	53.5	60.4	60.2	90.0‡	90.0‡	7.05	7
	" 3		9th	39.1	82.2	78.4	66.7	70.0	60.5	67.5	7.28	7
21	Mar. 12	Lobar pneumonia.	14th	38.9	81.1		45.6		46.5		7.23	
	" 14			37.9	88.5		48.1		44.5		7.27	
19	" 16	Lobar pneumonia. Pulmonary tuberculosis.	11th	39.0	87.5	78.6	40.4	41.2	30.0	31.5	7.38	7

* Case Nos. are the same as in Barach and Woodwell (10).

† A = Arterial; V = Venous.

‡ Approximate only.

H cted ygen atu- on.	pH corrected for oxygen unsaturation and for body temperature.		Remarks.
V	A	V	
7.41	7.42	7.42	Right lower lobe solid. Rest of chest fairly dry. Moderate dyspnea. No cyanosis. Recovered by crisis on 7th day.
7.28	7.27	7.31	Very sick, pneumonia (both lower lobes). Marked dyspnea. Marked cyanosis. Very poor quality pulse.
7.32	7.40	7.33	Still very sick. A little less dyspnea and cyanosis. Later developed empyema and died.
	7.27		Nearly moribund. Deep cyanosis (stagnant type of anoxemia). Rapid shallow breathing. Rapid feeble pulse. Died 2 hours after observation.
7.28	7.30	7.32	Very sick. Delirious. Moderate cyanosis and dyspnea. Died 6 hours after observation.
7.29	7.30	7.32	Very sick. Stuporous. Extreme hyperpnea. No cyanosis. Consolidation of left base, rest of chest dry.
7.50	7.53	7.52	Since yesterday given 30 gm. sodium bicarbonate with some relief to respiratory distress. 5 days later developed erysipelas and died.
	7.30		Fairly comfortable. Slight cyanosis. Moderate dyspnea. Consolidation of right lower lobe. Râles at left base.
	7.26		Had crisis on 9th day of disease. Now convalescent. 18 days since onset.
7.13	7.20	7.17	Moderately ill. No great respiratory distress. Slight cyanosis. Consolidation of right upper lobe, rest of chest clear.
	7.33		Recovered by crisis on 8th day of disease. Now well. Today is 25th day since onset.
.30	7.33	7.32	Very sick. Moderate dyspnea. Marked cyanosis. Râles in whole left chest. Consolidation of left base and below right clavicle.
.15	7.16	7.17	Seems in extremis. Outspoken generalized pulmonary edema.
.28	7.32	7.30	Better today. Improvement followed oxygen and alkali therapy. Later recovered from his pneumonia, but at home died of phthisis.
	7.26		Severely ill. Moderate dyspnea. Slight cyanosis.
	7.28		Crisis yesterday (15th day of disease). Very comfortable today. Slight dyspnea still. Later developed empyema. Operated. Recovered.
.40	7.42	7.42	Marked prostration. Moderate cyanosis and dyspnea. Consolidation of both lower lobes. Rest of chest clear. Later developed phthisis.

and the level of blood alkali. The former is shown by the position of the A- and V-points.

The pH of normal arterial blood as well as one can judge by the literature is not far from 7.35. Peters, Barr, and Rule (7) got this average for fully oxygenated blood of normal persons; their maximum was 7.42 and minimum 7.29. Roughly then, a pH between 7.30 and 7.40 may be considered normal. Now the average arterial pH of our ten sick pneumonia patients was as follows:

	pH
Uncorrected	7.26
Corrected for oxygen unsaturation	7.28
Corrected for oxygen unsaturation and for body temperature.	7.31

The range of variation is somewhat greater than in normal arterial blood, the maximum being (in the case of the doubly corrected values) 7.42 and the minimum 7.20. It would seem then on the basis of the corrected values for pH that there is no constant tendency toward an abnormal hydrogen ion concentration of the arterial blood in pneumonia. The average figure of 7.31 cannot be regarded as outside the normal range. Certain individual cases, however, seem to present figures somewhat lower than do any normals; for example, Nos. 14, 21, 22, and 20, with pH of 7.27, 7.26, 7.27, and 7.20 respectively. These results seem to indicate that in certain cases of pneumonia the hydrogen ion concentration actually is shifted in the direction of decreased alkalinity.

Bicarbonate Level.

The matter of the level of blood alkali is shown by the level of the dissociation curves. The extreme variations in the levels of the curves of normal persons are not known. In the previous paper a zone was shown within which all normal curves found in the literature (except those of Straub and Meier, 11) fell. This zone is shown again in the various figures of the present paper.

Peters, Barr, and Rule (7) show a very similar zone. Their lower border is essentially the same as ours, the upper border a little higher in order to contain the rather high level curves of the subject J. P. The levels of curves of pathologic blood in relation to the normal zone can be seen in the various figures. To

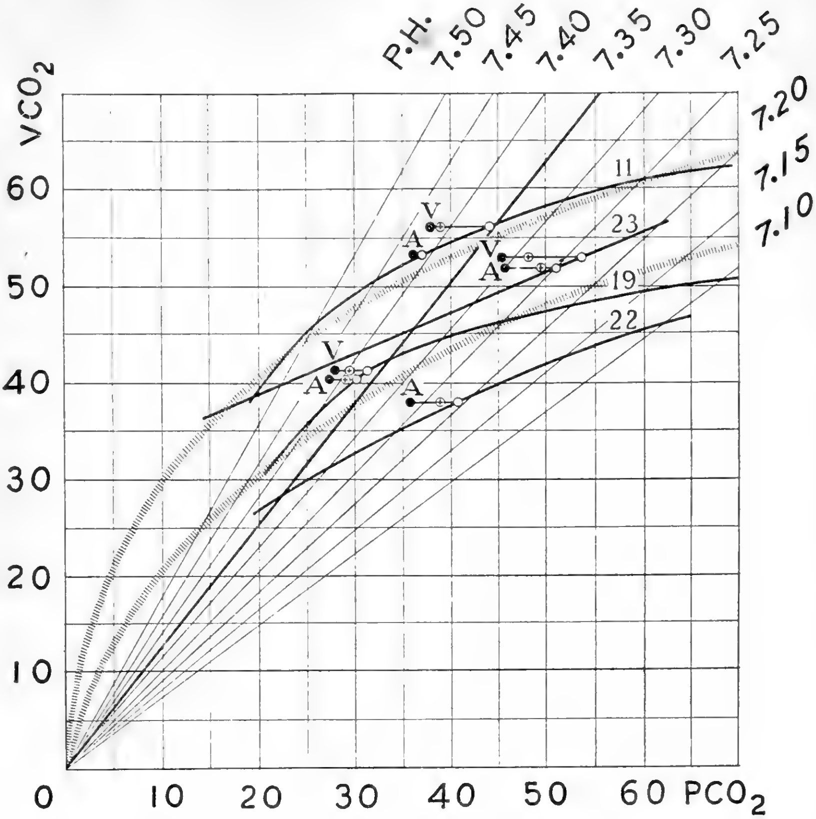

FIG. 1. Carbon dioxide diagrams in Cases 11, 19, and 23 (lobar pneumonia), and in No. 22 (bronchopneumonia).

In this and in all following figures the arterial points, A, and the venous points, V, are shown by circles. The points corrected for oxygen unsaturation are shown by circles containing crosses, and those corrected for body temperature as well by black dots.

The shaded zone indicates the area within which we believe the curves of normal blood should fall. P CO_2 = pressure of carbon dioxide in mm. of mercury. V CO_2 = carbon dioxide content of the blood in volumes per cent.

This figure shows three cases of lobar pneumonia with essentially normal diagrams, and one of bronchopneumonia with a slight depression in curve level and slight reduction in arterial pH.

For further data on these cases and also on those in following figures see Table I of this paper and also the paper of Barach and Woodwell (10).

measure actually the height of the curves we can read off the carbon dioxide content either with respect to isohydric points along, let us say, the pH 7.35 diagonal, or perhaps more conveniently for points of equal carbon dioxide tension, as for example along the 40 mm. ordinate. Peters, Barr, and Rule (7) found that at 40 mm. tension the average carbon dioxide content of all normal subjects of their own and from the literature (except those of Straub and Meier which they excluded for the same reasons as did Means, Bock, and Woodwell) was 49.3 volumes per cent, the maximum 55.9 and the minimum 43.3. The average carbon dioxide content of our ten sick pneumonia patients at 40 mm. carbon dioxide tension was 43.2 volumes per cent with a maximum of 54.5 and a minimum of 35.0. The pneumonia curves, therefore, show a lower average level than the normals, but also a greater range of variation. Half of them are essentially within normal limits, half somewhat lower than normal. From this we should conclude that in pneumonia there might either be a normal level of blood alkali or a slightly reduced one. A low level of blood alkali is according to Henderson's views (2) capable of one of two explanations, first as being due to non-volatile acidosis, second to acapnia. The latter is a condition of blood reaction more alkaline than normal, never of less alkaline; but our pneumonia diagrams show either a normal pH or one shifted in the direction of less alkalinity, hence those which show a low level curve must denote non-volatile acidosis rather than acapnia. The non-volatile acidosis when it occurs is slight; it is nothing like the marked lowering of blood alkali seen in diabetic or renal acidosis (see curves in first paper, 1).

To summarize our conclusions to this point we should say that the series of pneumonia bloods as a whole suggests that there may be at the height of this disease either a normal pH and level of blood alkali or that on the other hand in certain cases there may be an acidosis. This acidosis may be an actual non-volatile acidosis (but of slight degree only) as shown by a dissociation curve below the normal level, or it may be simply a carbon dioxide acidosis, that is to say no lowering of the dissociation curve but a change in blood reaction, as shown by the position of the A-point in the acid direction. This latter suggests that sometimes there may be insufficient pulmonary ventilation with a resulting re-

tention of carbon dioxide in the blood to an abnormal concentration. The two forms of acidosis may, theoretically at least, coexist.

The attempt was made to see whether the acid-base data furnished any clue to prognosis. None could be found except that all the fatal cases showed a pH of not over 7.30; but against this must be put the recovery of Case 20 who had the most marked acidosis of all, pH 7.20. Between carbon dioxide capacity at 40 mm. and death or recovery, there was no relation at all.

We also looked for possible relationships between pH and arterial oxygen saturation, and between carbon dioxide capacity at 40 mm. and arterial oxygen saturation, and again found none.

A further study of the individual cases brings out one or two points which may perhaps be of interest. In the first place the situation in pneumonia, if our interpretation of the findings is correct, is that when abnormalities exist they are acidoses, either non-volatile or carbon dioxide, or both. An appreciation of this situation introduces one or two clear-cut indications for treatment. We have recently discussed these indications in their broader aspects elsewhere (12). It will suffice here to point out that an individual suffering from respiratory embarrassment may theoretically be helped by having the level of his dissociation curve raised. This is true whether the curve is at a low level to start with or at a normal level but with a lowered pH. Raising a curve with a lowered pH may render the pH normal without change in carbon dioxide tension. This will mean that an insufficient pulmonary ventilation becomes efficient without increasing in volume. In other words, as brought out by Henderson and Haggard (13), it requires less ventilation to maintain normal hydrogen ion concentration at a given rate of carbon dioxide excretion with a high level of the dissociation curve than with a low one. To our minds, with two possible objections which we will discuss presently, it would seem desirable for the pneumonia patient with respiratory embarrassment to have the level of his dissociation curve raised.

Effect of Alkali Administration.

That the dissociation curve can be raised by the administration of sodium bicarbonate has been proved—the case of the nephritic

for example in the previous paper (1) or of Case 10 in the present (Fig. 7), and experimentally by Haggard and Henderson (2).

The two objections to alkali therapy which have been raised are these: In the first place, if, as the curve rose in level as alkali was given, the A-point passed to the left of the pH 7.35 diagonal, which it would do if a compensatory fall in pulmonary ventilation did not occur, we should be producing an alkalosis which in itself might be harmful. The second objection is one raised by Peters when we first reported this work, and that is that raising the curve and diminishing the pulmonary ventilation in pneumonia might be harmful because it would produce or increase an existing anoxemia.

Whether the phenomenon mentioned in the first objection occurs with any regularity we do not know. It did happen to a certain extent in the nephritic already referred to and in Case 17 of the present paper (Fig. 6), in neither instance, however, with any apparent ill effects that could be attributed to alkalosis. In Case 10, however, a marked rise in the curve occurred with no alkalosis developing. It seems to us that the likelihood of producing a dangerous alkalosis is slight, particularly if the reaction of the urine is carefully followed and alkali administration stopped at once upon its becoming alkaline.

Peters' objection is a perfectly valid one. Anoxemia does often exist in pneumonia. Decreasing pulmonary ventilation might aggravate it. Giving alkali to diminish pulmonary ventilation, therefore, might be the diametrically wrong thing to do in pneumonia. The answer to the objection is, of course, that we should not only raise the dissociation curve by alkali administration but at the same time abolish anoxemia by oxygen administration. That anoxemia in pneumonia can be relieved or abolished by oxygen therapy has been proved in this clinic (10) and by others (14).

Arterial and Venous Blood in Pneumonia.

Another matter on which the present studies may throw some light is that of the relation between the reaction of arterial and venous blood in pneumonia. Peters, Barr, and Rule have already discussed this relationship in the bloods of normal persons. They state their findings in three normal subjects as follows:

"The CO_2 tension of venous blood was found to vary between 42 and 72 mm. uncorrected for oxygen unsaturation, 39.5 to 58.5 mm. after correction, with an average of 50.2 mm. The corresponding values for pH were 7.37 to 7.12 uncorrected, 7.40 to 7.22 corrected, with an average of 7.31."

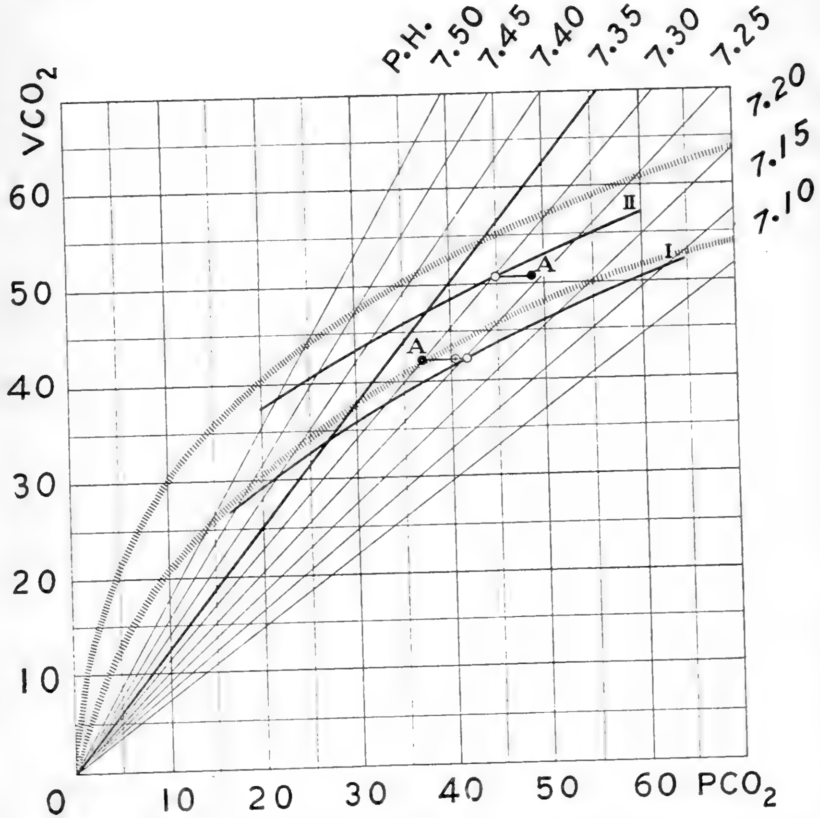

FIG. 2. Carbon dioxide diagrams in Case 13 (lobar pneumonia), I, on the 5th day of the disease and II, 9 days after the crisis, which was on the 9th day of the disease.

In this case a curve just below the normal limit assumes a normal level after the crisis.

The effect of oxygen saturation on carbon dioxide-combining power is exerted in the direction of keeping blood reaction nearly constant, of their three normals the average corrected arterial

and venous pH both being 7.31. Taking out of our ten sick and untreated pneumonia cases the seven in which we have observations on both arterial and venous blood, we find an average pH corrected for oxygen unsaturation of 7.29 for both arterial and venous blood, and corrected for body temperature 7.32. Even in the four most acidotic of these bloods we find an average arterial pH, corrected for oxygen unsaturation and body temperature, of 7.27, and for the venous 7.28. In pneumonia then (even in the presence of acidosis) there is, as there is in normal persons, little if any difference in pH between arterial and venous blood.

Effect of the Crisis.

In considering those cases of our series in which we have more than one curve, certain interesting features appear. In three cases for example (Nos. 13, 20, and 21) we have observations before and after the crisis. In each of these the second observation showed a higher level of the curve than the first, thus:

Case No.	CO_2 content at 40 mm.		Rise in level of curve.
	Before crisis.	After crisis.	
	vol. per cent	*vol. per cent*	*vol. per cent*
13	41.3	48.3	7.0
20	35.0	53.2	18.2
21	41.3	45.3	4.0
Average			9.7

The pH of the arterial blood (corrected for oxygen unsaturation and for body temperature) of these same patients before and after crisis was as follows:

Case No.	Arterial pH.	
	Before crisis.	After crisis.
13	7.30	7.26
20	7.20	7.33
21	7.26	7.28
Average	7.28	7.29

In these three cases therefore, there was seen following a crisis a return of the blood acid-base balance toward normal in two directions. The available alkali as shown by the curve levels lower than normal before crisis in all three (slightly in Nos. 13

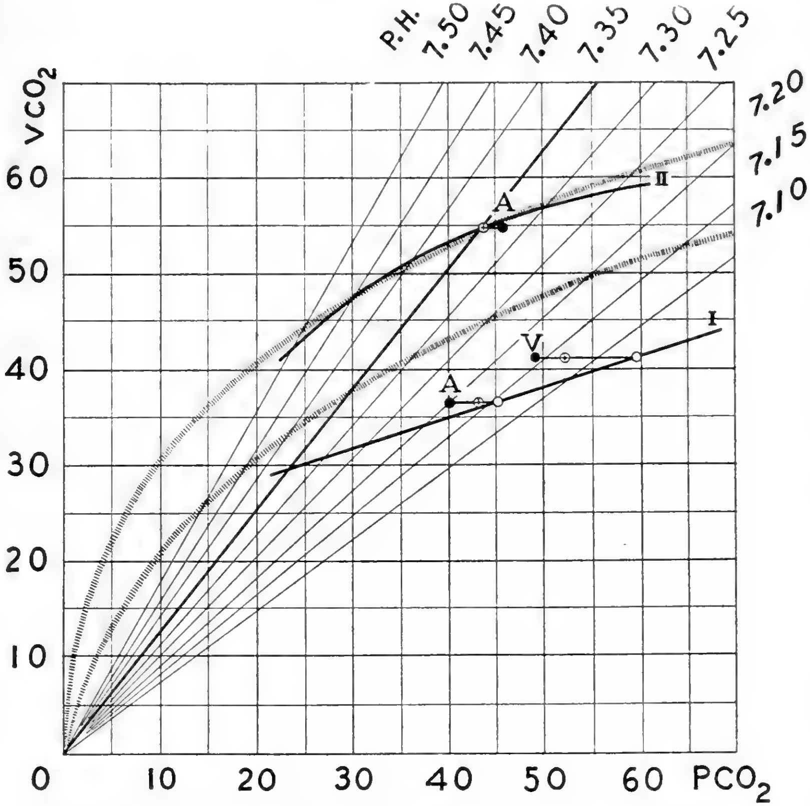

FIG. 3. Carbon dioxide diagrams in Case 20 (lobar pneumonia), I, on the 7th day of the disease and II, 17 days after the crisis, which was on the 8th day of the disease.

This case showed a marked rise in curve level from a low position to a high normal position after the crisis and a rise in pH from 7.20 to 7.33.

and 21 and definitely in No. 20) in each instance rose to an entirely normal level after the crisis. This is most marked in Case 20, which is not surprising as this patient had the most acidosis to start with and also in this case the second observation

was 17 days after the crisis, while in No. 13 the second observaions was only 9 days after crisis and in No. 21 only 1 day.

The hydrogen ion concentration in two of the three cases showed a tendency to move from a less alkaline reaction than normal

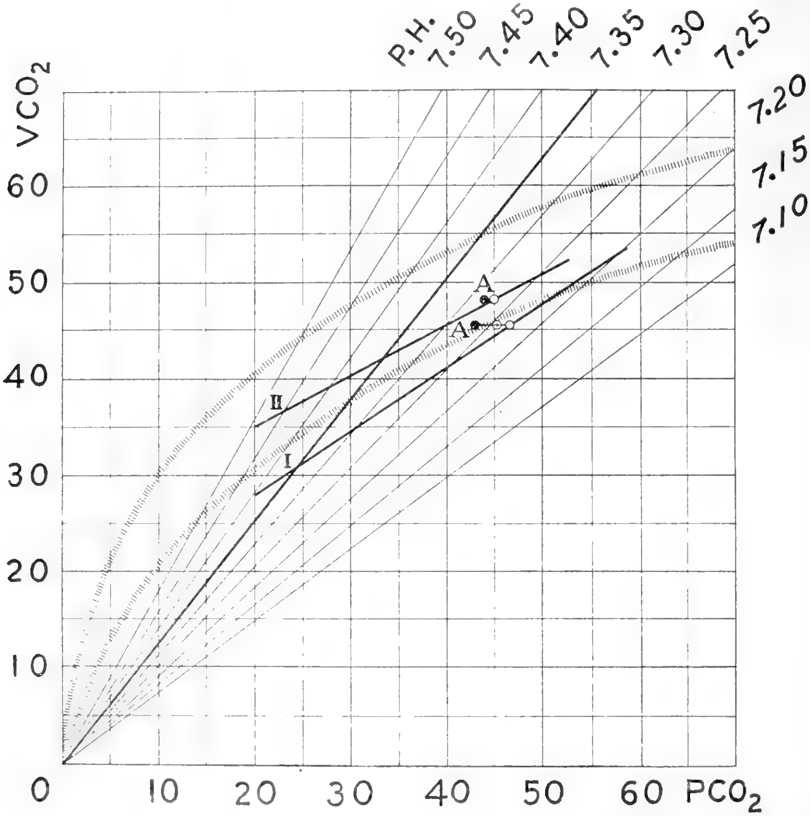

FIG. 4. Carbon dioxide diagrams in Case 21 (lobar pneumonia), I, day before crisis, II, day after crisis. The crisis was on the 15th day of the disease.

This case shows a slight rise in curve level the day after the crisis.

toward a normal one after crisis. This phenomenon like the other is more noticeable in Case 20, the one most acidotic to start with.

The changes undergone by the blood in these three cases before and after the crisis are shown diagramatically in Figs. 2, 3, and 4.

The effect on the acid-base balance of the two therapeutic measures which as suggested above and elsewhere (12), seem to us on theoretical grounds often indicated, either alone or coin-

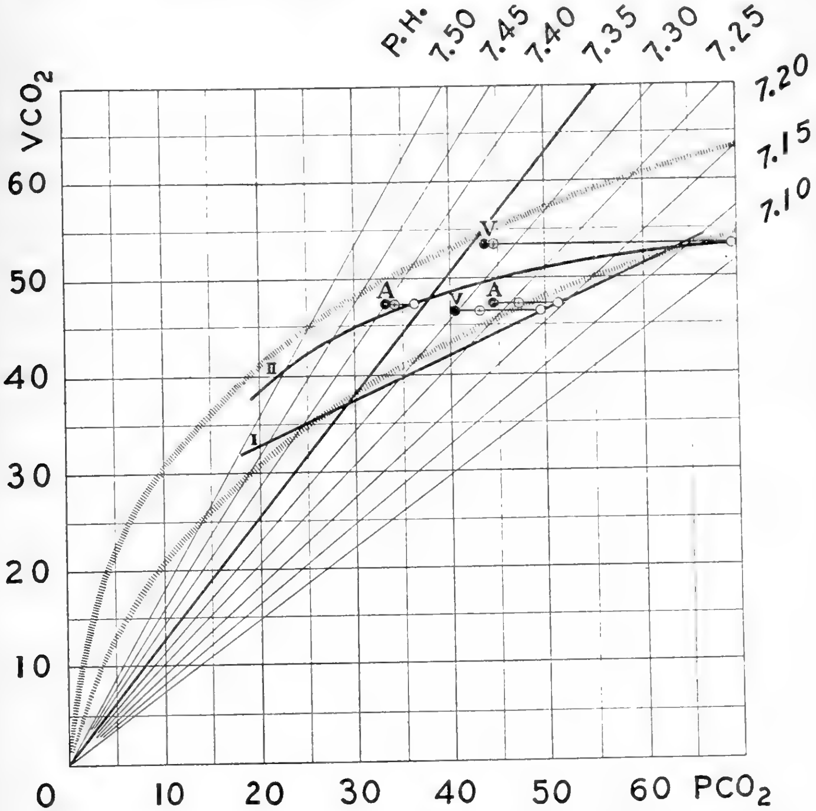

FIG. 5. Carbon dioxide diagrams in Case 14 (lobar pneumonia), I, on 7th day and II, on 9th day of disease. Between Curves I and II he was treated intensively with oxygen.

Here a slight rise in curve level and pH followed oxygen therapy but with no crisis.

cidently, namely alkali and oxygen therapy, also receive some light from the present research.

The outstanding effect of oxygen therapy is, of course, relief of anoxemia. This has been discussed in detail in other papers

(10, 12), but it is theoretically at least conceivable that relief of anoxemia may have in itself some beneficial effect upon acidosis. The recovery of normal blood alkali level just described, which

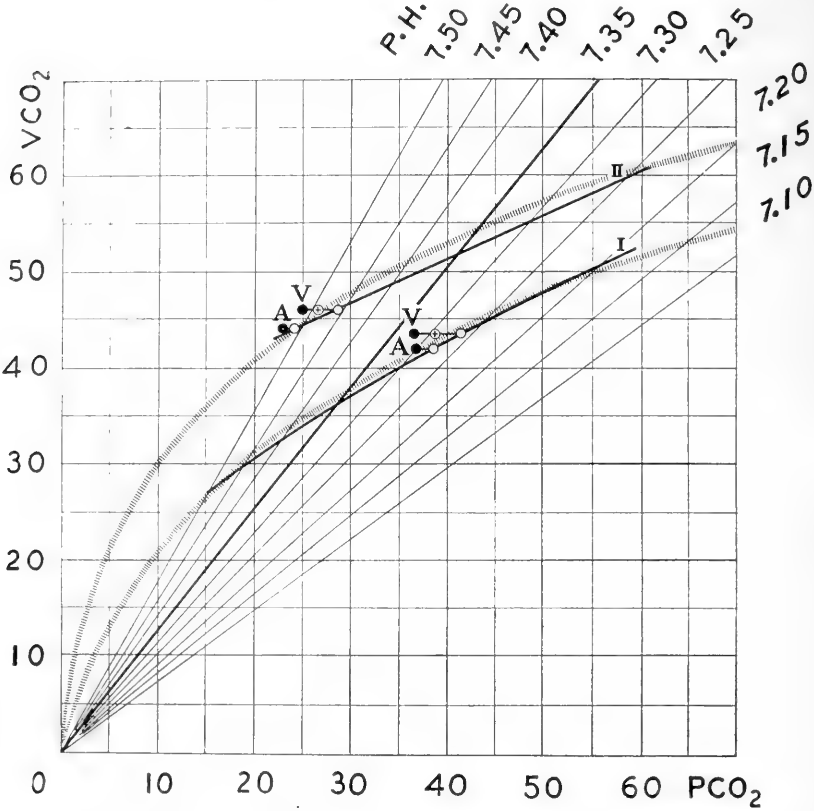

FIG. 6. Carbon dioxide diagrams in Case 17 (bronchopneumonia), I, on the 21st and II, on the 22d day of the disease. Between the two he received 30 gm. of sodium bicarbonate.

In this case a rise in curve level followed alkali administration and the pH was shifted to the alkaline side of normal, an alkalosis was produced but no harmful signs or symptoms of that condition appeared.

occurs after crisis, could in the three cases under discussion hardly have been due to oxygen therapy for no one of these received oxygen except for a short period. Case 14, however, in which we have two sets of data 2 days apart, showed a rise in curve level

and return of blood reaction from an acidotic to a normal one although he had had no crisis and on the second observation was still very ill though less dyspneic. He eventually died of empyema. The results in this case are shown in Fig. 5. From the

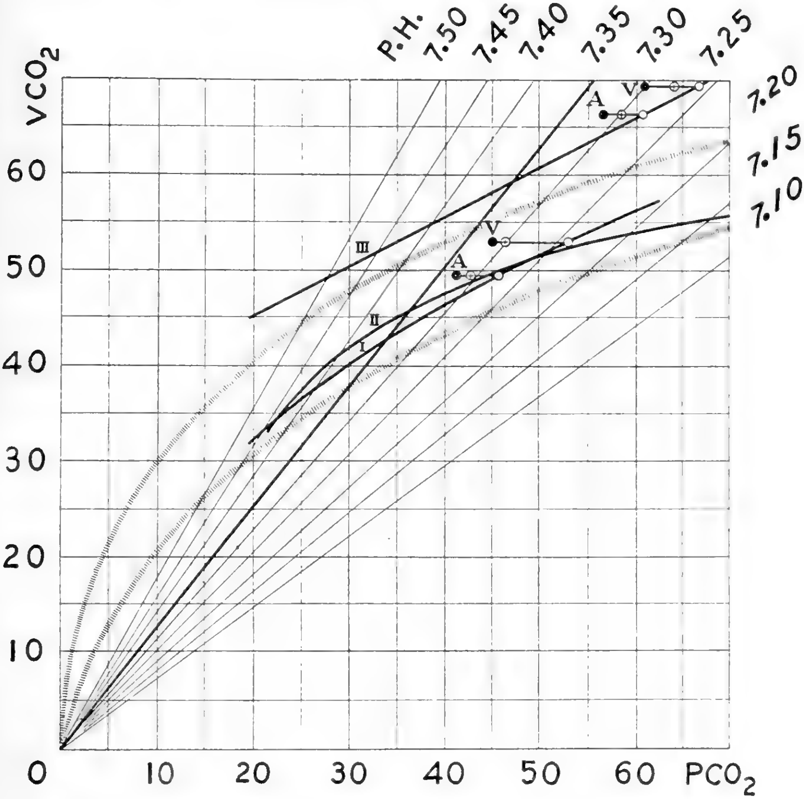

FIG. 7. Carbon dioxide diagrams in Case 10 (lobar pneumonia), I, on the 6th, II, on the 8th, and III, on the 9th day of the disease. Between Curves II and III he was treated with oxygen and was given 15 gm. of sodium bicarbonate.

A marked rise in curve level without change in pH resulted.

time of the first curve on the 7th day of the disease to that of the second curve on the 9th day, the arterial oxygen saturation rose from 74.4 to 91.6 per cent, due, it was believed, to the vigorous oxygen therapy which he received. As to whether the rise in

curve level from 41.8 volumes per cent at 40 mm. to 48.5, and that of corrected arterial pH from 7.27 to 7.40, were in any way the result of oxygen therapy one can only speculate. The problem, however, in that respect is worthy of further study.

The effect of alkali administration was studied in two cases. In Case 17 (Fig. 6), between the first and second curves 30 gm. of sodium bicarbonate were given by mouth. Probably as a result of this, for there was no crisis, the level of the curve rose from 42.5 volumes per cent at 40 mm. to 51.3, and the corrected arterial pH from 7.30 to 7.53. In this case an actual alkalosis was produced, but no harmful effects due to that were noted and the dyspnea which had been present at the start was somewhat relieved.

In Case 10, shown diagrammatically in Fig. 7, three curves were obtained. The first of these on the 6th day of the disease showed a curve at a normal level and perhaps a very slight carbon dioxide acidosis. On the 8th day of the disease the curve was essentially the same as to level. No satisfactory A-point was obtained. Between the time Curve II was obtained and the next day when Curve III was obtained he was treated intensively with oxygen and was also given 15 gm. of sodium bicarbonate, the dissociation curve showed a rise of 8 volumes per cent at 40 mm. and at the same time some relief in respiratory distress.

SUMMARY.

1. Carbon dioxide diagrams of the bloods of ten new cases of pneumonia are presented. In three cases observations were secured before and after the crisis, in one case before and after oxygen therapy, and in two cases before and after the administration of sodium bicarbonate.

2. The alkali of the blood in pneumonia as shown by the level of the carbon dioxide dissociation curve, that is to say by the carbon dioxide capacity at a fixed carbon dioxide tension (40 mm.), was found to be sometimes within normal limits, sometimes somewhat below normal limits. The average in the pneumonia group was 43.2 volumes per cent, while in normal persons Peters, Barr, and Rule found the average 49.3 volumes per cent. The lowest observed in pneumonia was 35.0 volumes per cent.

3. The arterial pH in pneumonia as calculated from the carbon dioxide diagram and corrected for oxygen unsaturation and body temperature showed an average of 7.31. Four of the ten bloods showed a pH below 7.30, which probably can be considered the lower border of normal variation. The lowest observed was 7.20.

4. No relation between pH or dissociation curve level and degree of anoxemia or prognosis could be found.

5. In pneumonia patients, as in normal persons, there seems to be little or no difference in pH between arterial and venous blood.

6. In three patients studied before and after crisis there was an increase in curve level after crisis in each instance, and in one showing a marked shift in pH before crisis there was a normal pH after crisis. A rise in curve level and a return to a normal pH in the cases with lowered pH would seem to be among the phenomena that take place at or after the crisis.

7. The same phenomena occurred in one case without crisis but after vigorous treatment with oxygen.

8. In two cases the level of the curve was raised apparently by the administration of sodium bicarbonate, in one instance with the production of a slight alkalosis.

9. It is suggested that in pneumonia patients showing acidosis either in the sense of a low level of available blood alkali or of decrease in pH or combination of the two, the administration of sodium bicarbonate may be helpful by diminishing the work of the respiratory bellows. By such a procedure a pH less alkaline than normal may be brought to normal with no increase in ventilation because of a raising in the level of the dissociation curve. Or in a case with low curve but normal pH to start with, the raising of the curve may diminish the amount of ventilation necessary. The use of sodium bicarbonate should be carefully controlled, however, to avoid the production of alkalosis, and when anoxemia is present should be combined with oxygen therapy.

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ANALYSIS AND COMPOSITION OF CORN POLLEN.

PRELIMINARY REPORT.

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INTRODUCTION.

The literature dealing with corn and corn products is very extensive but we have failed to find in it any reference to the composition of corn pollen. In the process of fertilization and reproduction pollen plays a very important part. It would seem, therefore, that some knowledge of the kind and nature of the chemical compounds occurring in pollen would be of interest to plant physiologists. Different varieties of corn apparently produce pollen which varies greatly in composition. This fact might be of importance in cross-breeding.

The present investigation was undertaken in order to contribute some information on the following points: (1) The approximate composition of corn pollen, (2) the principal inorganic constituents of the ash, and (3) the principal organic compounds contained in the pollen grains.

Unfortunately, stress of other work has prevented us from completing the investigation, but we wish to publish this preliminary report giving the results which have been obtained up to the present time because our joint work will be interrupted during the coming year.

EXPERIMENTAL.

The pollen was gathered in the following manner: Corn tassels were cut off as the pollen sacs were opening. The tassels were spread out in thin layers on clean paper on the floor and allowed to dry. The pollen was then shaken out on clean paper and care-

fully sifted. Many of the pollen sacs did not open and in order to obtain the enclosed pollen it was necessary to break them by rubbing or by passing the tassels through a coarse mill and then carefully sifting out the pollen. Practically all foreign matter was finally removed by sifting through very fine bolting cloth.

It is important that fresh pollen be spread out in thin layers while drying as otherwise it will undergo very rapid decomposition. One day about 100 gm. of ripe pollen obtained by shaking fresh corn tassels over paper were left over night in a dish which was loosely covered by a watch-glass. The pollen was a heavy yellow powder which appeared to be dry. The next morning it was found to consist of a semifluid gummy mass in which it was impossible to distinguish any individual pollen grains. We were unable at that time to make any investigation of the changes which had occurred and this observation is merely recorded to indicate the rapidity with which pollen may undergo spontaneous decomposition when conditions of temperature and moisture are favorable.

In 1919 we obtained about 800 gm. of pollen from yellow dent Improved Leaming corn, and in 1920 some 2,000 gm. of pollen were gathered from White Flint Luces Favorite and a smaller quantity from pop-corn.

The pollen obtained as outlined above formed a golden yellow, dense powder and had a strong but agreeable aromatic odor. 100 gm. of pollen occupied about 150 cc. of space.

Determination of Moisture.

On drying at 103°C. the pollen continued to lose in weight slowly and after 48 hours the loss amounted to about 6.5 per cent. All of this loss in weight was not due to loss of water because the dried pollen was dark brown in color and had lost practically all of the characteristic odor. After drying at 100°C. the color darkened and most of the odor was lost. It is evident, therefore, that in drying at these temperatures certain volatile constituents are lost and that some oxidation occurs.

In order to obviate such losses of volatile principles and to prevent oxidation the pollen was dried for analysis at room temperature in vacuum over sulfuric acid. The loss in weight on drying in this manner was 4.68 per cent and there was no noticeable change either in color or odor.

Extraction of the Pollen with Various Solvents.

A series of extractions was made to determine the amounts of material removed from the pollen by ether and alcohol during varying lengths of time. The results obtained are given in condensed form in Table I. The percentages are all calculated to the original air-dry pollen.

TABLE I.

Ether and Alcohol Extraction of Corn Pollen.

Ether extraction; Soxhlet method.				Alcohol extraction following ether extraction.				Amount of alcohol extract soluble in ether.
Extraction.	Pollen used.	Weight of extract.	Percentage of extract.	Extraction.	Pollen used.	Weight of extract.	Percentage of extract.	
hrs.	*gm.*	*gm.*	*per cent*	*hrs.*	*gm.*	*gm.*	*per cent*	
8	5	0.0538	1.07	4*	5	1.6166	32.33	14.93
24	10	0.1320	1.32	24 (Soxhlet)	10	1.1405	11.40	
48	20	0.3170	1.58	168 (Soxhlet)	20	2.9272	14.63	
48	2	0.0280	1.40	4 weeks.†	20	6.8267	34.13	
48	2	0.0285	1.42					

* 5 gm. of pollen were suspended in 75 cc. of absolute alcohol and boiled under reflux condenser for 1 hour on the water bath. The alcoholic solution was decanted and replaced by fresh alcohol which was in turn boiled for 1 hour. These operations were repeated four times. The alcoholic extracts were united, filtered, and evaporated to dryness and then dried in vacuum over sulfuric acid.

† After extracting 20 gm. of pollen with absolute alcohol in a Soxhlet apparatus for 7 days, the pollen residue was suspended in about 100 cc. of absolute alcohol and boiled on the water bath under reflux condenser. The alcohol was decanted and renewed daily until all of the coloring matter was removed and the alcohol remained practically colorless. The time required was 4 weeks. The alcoholic extracts were united, the alcohol was distilled off, and the extract was dried in vacuum over sulfuric acid.

It is interesting to note that extracting pollen with four portions of boiling absolute alcohol yields almost as high a percentage of extract as was obtained after completely exhausting the pollen with absolute alcohol during a period of 4 weeks.

A more complete extraction was obtained when the pollen was suspended in the alcohol in a small flask and digested under a reflux condenser on the water bath than when the pollen was contained in a thimble as in the usual Soxhlet method.

The dried ether extract was of a dirty green color and of a rather soft, wax-like consistency. This extract contained only a trace of phosphorus and consequently it could only contain a very small amount of phosphatide. The nature of this fat or wax-like material has not been determined.

Extraction of Corn Pollen with Absolute Alcohol and Chloroform.

It has been stated by Glikin (1) that the method of Rosenfeld (2) gave high yields of fat and lecithin, particularly in the analysis of animal tissues. This method consists in extracting the material for $\frac{1}{2}$ hour in boiling alcohol and then extracting the residue for 6 hours with chloroform in a Soxhlet apparatus.

We employed this method on corn pollen as follows: (*a*) 5 gm. of pollen after drying in vacuum over sulfuric acid, were placed in an extraction thimble and extracted for 2 hours by immersing in about 50 cc. of boiling absolute alcohol contained in a large test-tube. The thimble and contents were then rinsed with absolute alcohol. The solution was filtered, the alcohol evaporated, and the extract dried to constant weight in vacuum over sulfuric acid. The dry extract weighed 0.3815 gm. or 7.63 per cent of the air-dried pollen. (*b*) The pollen residue was extracted for 6 hours with chloroform in a Soxhlet apparatus. After evaporating the chloroform and drying as above the extract weighed 0.4215 gm. or 8.43 per cent. (*c*) The pollen residue was extracted a second time with chloroform for 6 hours. After evaporating and drying as above the extract weighed 0.0538 gm. or 1.08 per cent. (*d*) The pollen residue was extracted a third time with chloroform for 48 hours. On evaporating and drying as before there was obtained a semicrystalline material which weighed 0.0340 gm. or 0.68 per cent.

The total yield of extract in the above operations was, therefore, 0.8908 gm. or 17.82 per cent. These extracts were united and exhausted with absolute ether. After filtering, evaporating the ether, and drying, the ether-soluble extract weighed 0.6940 gm. or 13.88 per cent.

The ether-insoluble material which remained was a semicrystalline solid which was readily soluble in water. Evidently, therefore, it was neither fat nor lecithin.

Attention is called to the fact that a higher yield of extract was obtained by digesting the pollen in four successive portions of alcohol as shown in Table I than by the above alcohol-chloroform extraction. The ether-soluble part of the alcoholic extract was 14.93 per cent as against 13.88 per cent of alcohol-chloroform extract. The lecithin content of the ether-soluble portion of the alcoholic extract was also higher than in the alcohol-chloroform extract as will be shown below. But only a small amount of the ether-soluble material was phosphatides as shown by the low phosphorus content. The nature of the non-phosphatide part of the ether-soluble portion of the alcoholic extract has not been determined.

It is interesting to note that while the maximum amount of ether extract obtained by direct extraction of the pollen with absolute ether was only 1.58 per cent yet the amount of alcohol-chloroform and the absolute alcohol extracts soluble in absolute ether was from 14 to 15 per cent of the weight of the pollen.

It is probable that the membrane surrounding the pollen grains is nearly impermeable to ether while alcohol and chloroform permeate the membrane and dissolve out fats and phosphatides together with other substances. This assumption would account for the low percentages of ether-soluble material obtained in the direct extraction of the pollen with ether and for the much larger yields of ether-soluble substances in the alcoholic or chloroform extracts.

The difficulty of completely extracting the soluble constituents is greater in the case of pollen than in other plant material because it is practically impossible to rupture the pollen membranes by ordinary trituration. Prolonged grinding in a mortar, even after the pollen has been extracted with ether and alcohol, produces only a small percentage of broken cells.

Phosphatide Content of Corn Pollen.

The absolute ether extract obtained from pollen contained, as previously stated, only a trace of phosphorus. A larger amount of ether-soluble or phosphatide phosphorus was contained in the alcoholic and the alcohol-chloroform extracts.

The phosphorus in the alcoholic extract amounted to 0.19 per cent of the pollen. Nearly all of this phosphorus, or 0.139 per

cent was soluble in ether. Multiplying this number by the usual factor for lecithin we obtain 3.62 per cent of lecithin in pollen. In the alcohol-chloroform extract the ether-soluble phosphorus amounted to 0.104 per cent which corresponds to 2.72 per cent of lecithin. These figures indicate that the phosphatides are more completely removed from pollen by absolute alcohol than by the alcohol-chloroform treatment.

Nitrogen in Corn Pollen and the Nitrogen Distribution in Pollen Extracts.

The total nitrogen in the air-dried pollen was 4.30 per cent.

10 gm. of pollen were extracted with ether in a Soxhlet apparatus for 24 hours. It was then extracted with absolute alcohol for 24 hours. The alcohol was evaporated and the extract was taken up in ether as much as possible, filtered, and the ether evaporated.

The ether extracts were united and were found to contain 0.14 per cent of nitrogen.

The ether-insoluble portion of the alcoholic extract contained 0.18 per cent of nitrogen.

The pollen residue after extracting with ether and alcohol was digested in water, filtered, and washed with water. The water-soluble nitrogen amounted to 0.50 per cent. The pollen residue from the above extractions, after drying contained 3.49 per cent of nitrogen.

The above results are calculated to the original air-dried pollen.

Approximate Composition of Corn Pollen.

The figures given in Table II were obtained on analyzing the pollen obtained from three varieties of corn. The results are calculated to the water-free pollen.

We do not feel that we can give any adequate reason for the striking differences found for starch and sucrose. Several determinations were made in duplicate and triplicate with concordant results. It is not impossible that the difference in composition depends upon varying degrees of ripeness but we tried, as far as possible, to gather all of this pollen when it was just ripe.

It is not improbable that different varieties of corn may produce pollens of different composition. However, until more work

has been done on this subject we would only offer this explanation with some reserve.

TABLE II.

Analysis of Corn Pollen.

Constituent.	Yellow dent corn. Improved Leaming. Pollen gathered 1919.	White flint corn. Luces Favorite. Pollen gathered 1920.	Pop-corn. Pollen gathered 1920.
	per cent	*per cent*	*per cent*
Starch	11.07	19.04	18.03
Nitrogen	4.53	4.43	3.85
Reducing sugar as dextrose	3.50	5.38	4.95
Sucrose	9.09	2.97	14.18
Pentosans	10.60		
Crude fiber	5.35		
Crude fat or ether extract (average)	1.48		
Ash	3.46	3.83	3.13
Phosphorus	0.63		
Sulfur	0.34		
Chlorine	0.19		
Potassium	1.24		

Analysis of the Pollen Ash.

For analysis the pollen was ashed at a low temperature in an electric muffle and a pure white ash was obtained. The result of the analysis is given in Table III.

TABLE III.

Composition of the Pollen Ash from Yellow Dent Improved Leaming Corn.

Constituent.	Per cent.
Phosphorus	18.92
Sulfur	0.69
Chlorine	0.80
Silica, SiO_2	3.76
Calcium	1.02
Magnesium	4.60
Potassium	35.58
Sodium	0.69
Iron	0.25
Aluminum	0.22

Separation of Certain Soluble Constituents of Corn Pollen.

The pollen used in this investigation was obtained from the Improved Leaming variety of yellow dent corn which had been gathered in 1919. It was dried in vacuum over sulfuric acid.

Extraction with Ether.

The pollen, 590 gm. of dry material, was extracted with absolute ether during two 24 hour periods. After evaporating the ether and drying the extract in vacuum over sulfuric acid it weighed 9.0 gm. This is equal to 1.52 per cent of ether extract. The extract was of a dirty green color and of a soft, wax-like consistency. It was not further investigated.

Extraction with Alcohol.

The pollen residue was placed in a 2 liter flask, 1 liter of absolute alcohol was added and the mixture was heated to 60°C. under a reflux condenser for 1 hour. It was allowed to stand at room temperature for 15 hours and then heated to 60° for about 5 hours. It was filtered while hot on a Buchner funnel and washed with absolute alcohol. These operations were repeated three times with fresh portions of alcohol. The pollen residue was reserved for further examination.

Examination of the Alcoholic Solution.

The alcoholic extract was of a greenish yellow color and it measured about 4 liters.

On cooling and standing over night in the ice chest there separated out a small amount of colorless crystals in the bottom of the flask. This material was filtered off and will be referred to later as "Substance A."

The alcoholic solution was concentrated in vacuum at a temperature not exceeding 40° to about 300 cc. During the evaporation of the alcohol a considerable amount of a crystalline substance was deposited in the flask and the quantity increased on cooling and standing over night. The crystals were filtered off and washed in alcohol. This material will be referred to later as "Substance B."

The alcoholic solution was now taken to dryness in vacuum. There remained a thick oily substance which was mixed with some crystalline product. This oily residue was shaken with several portions of absolute ether in which the greater amount of the material dissolved. The ether-insoluble, semicrystalline substance was added to "Substance B" mentioned above.

Preparation of the Amorphous Phosphatide.

The ethereal solution was evaporated to a syrupy consistency and to it were added with constant shaking 600 cc. of acetone. A heavy sticky substance was precipitated which settled to the bottom of the flask. The dark-colored acetone solution was decanted and the residue was washed thoroughly with acetone.

The acetone solution and washings were concentrated to a thin syrup and again poured into 600 cc. of acetone when a further quantity of the sticky substance, similar to the first, separated. After decanting the mother liquor and washing with acetone this precipitate was added to the first amorphous phosphatide.

Preparation of the Crystalline Phosphatide.

The acetone mother liquor was allowed to stand in the ice chest for 2 days. A considerable quantity of nearly colorless, large, thin, plate-shaped crystals separated gradually. The crystals were removed, washed in acetone, and dried in vacuum over sulfuric acid. The dry substance weighed 4 gm. This crystalline material was found to be a phosphatide. It will be described later.

The acetone solution was evaporated to dryness under reduced pressure. There remained a thick oily brown residue which, after drying in vacuum over sulfuric acid, weighed 26 gm. This material still contained a considerable amount of phosphatide because it contained 0.55 per cent of phosphorus and 0.4 per cent of nitrogen, but it was not further examined.

Purification of the Amorphous Phosphatide.

The substance which was precipitated by acetone from the ethereal solution was dissolved in absolute ether. The ether solution was shaken with water and afterwards with a dilute solu-

tion of sodium chloride. The emulsions which formed were broken up with much difficulty by adding sodium sulfate. The solution was finally dried with sodium sulfate, filtered, and the ether evaporated until a thin syrup remained. This was poured with constant stirring into 600 cc. of acetone. The phosphatide separated as a thick, pasty mass. The acetone was decanted and the phosphatide washed several times by thoroughly stirring with acetone. After drying in vacuum over sulfuric acid it weighed 11.5 gm.

The two phosphatide preparations had a combined weight of 15.5 gm. This corresponds to a yield of 2.6 per cent.

The amorphous phosphatide after drying formed a light yellowish brown, hard, brittle mass which could be powdered. It was not very hygroscopic. For analysis it was dried in vacuum over phosphorus pentoxide at the temperature of boiling chloroform. Further drying at 78°C. did not cause any loss in weight. There was no perceptible change in color on drying at the above temperature and the loss in weight was only 1.09 per cent.

Found. P = 3.86, N = 1.53 per cent.
Ratio N : P = 1 : 1.1

The percentage of phosphorus and nitrogen and the N:P ratio corresponded very nearly to the values required for distearyl lecithin.

Hydrolysis of the Amorphous Phosphatide.

Without subjecting the substance to any further purification an attempt was made to determine quantitatively the amounts of choline, glycerophosphoric acid, and fatty acids after hydrolysis. In this experiment we followed the method outlined by Osborne and Wakeman (3) in their study of the hydrolysis of the phosphatide from milk. We used 5.2390 gm. of the dry phosphatide and obtained 0.8519 gm. of choline platinum chloride, 1.2035 gm. of barium glycerophosphate, and 2.2646 gm. of fatty acids. The figures presented in Table IV are calculated from the above values.

The choline platinum chloride after recrystallizing from water contained 32.21 per cent of Pt.

$(C_5H_{14}ONCl)_2PtCl_4$. Calculated. Pt 31.64 per cent.

The barium glycerophosphate was purified by precipitating it from aqueous solution with alcohol until a pure white amorphous preparation was obtained. The air-dried substance lost 8.30 per cent of water on drying at 105°C. in vacuum over phosphorus pentoxide and the weight remained constant on further drying at 130°C. On analysis the dried preparation gave:

Ba = 39.90, P = 9.17 per cent.
$C_3H_7O_6P$ Ba + 2 H_2O. Calculated. Ba = 40.01, P = 9.02 per cent.

The analytical results agree with the theoretical composition of barium glycerophosphate plus 2 H_2O. But the fact that this water could not be driven off at 130°C. in vacuum makes the purity of the preparation somewhat doubtful. Winterstein and Hiestand (4) obtained a barium glycerophosphate of similar com-

TABLE IV.

Cleavage Products of Amorphous Phosphatide.

Constituent.	Amount found.		Calculated for distearyl lecithin.
	gm.	*per cent*	*per cent*
Choline	0.3335	6.36	14.99
Glycerophosphoric acid	0.6733	12.85	21.31
Fatty acids	2.2646	43.22	70.26

position from the phosphatide which they had isolated from wheat flour. MacLean (5) calls attention to the difficulty of purifying the glycerophosphoric acid prepared from plant phosphatides.

Some evidence was found of the presence of another base besides choline in the phosphatide. After the choline platinum chloride had been filtered off the alcoholic solution was evaporated and the residue was taken up in water. The platinum was precipitated by hydrogen sulfide and the filtrate was evaporated to dryness under reduced pressure. The residue was extracted at room temperature with absolute alcohol which left a small quantity of an insoluble white crystalline substance. This was recrystallized from hot 95 per cent alcohol and was obtained in colorless needle-shaped crystals which weighed 0.13 gm. From this substance a gold double salt was prepared which crystallized from

water in large yellow needles. It contained 49.84 per cent of gold and melted at 132°C. (uncorrected), but we were unable to identify this substance.

On hydrolysis of the phosphatide a mixture of saturated and unsaturated fatty acids was obtained. The percentage of iodine absorbed by the crude fatty acids was 49.01 determined by the Hanus method (6). The fatty acids were saponified and lead soaps were prepared and extracted with ether. The ether-insoluble lead salt was decomposed with hydrochloric acid and extracted with ether. After evaporating the ether, the residue was recrystallized several times from absolute alcohol. The snow-white crystals melted at 63°C. (uncorrected). The melting point of palmitic acid is 62.6°C. and it is probable, therefore, that the saturated acid was nearly pure palmitic acid.

Owing to the small quantity of unsaturated acid its nature could not be determined.

The values found for the cleavage products of the amorphous phosphatide as indicated in Table IV are very much lower than is required for the formula of distearyl lecithin. This might be due to admixed impurities or possibly to the presence of carbohydrates. An attempt was made to determine the amount of carbohydrate present in the phosphatide. After hydrolyzing by boiling with 5 per cent sulfuric acid, as described by Winterstein and Hiestand (7), cooling, and neutralizing with sodium hydroxide only a very slight reduction was obtained on boiling with Fehling's solution. The phosphatide contained, therefore, only a trace of carbohydrate.

A complete analysis of the substance was made when it was found that, in addition to carbon, hydrogen, phosphorus, and nitrogen, it also contained sulfur. The results obtained on analysis are given below.

Found. C 57.78, H 8.53, P 3.86, N 1.53, S 0.68 per cent.

The carbon and hydrogen are much lower than is required for distearyl lecithin and the presence of sulfur would indicate that the substance is a mixture of phosphatide and sulfatide.

Analysis of the Crystalline Phosphatide.

The crystalline phosphatide which separated from the acetone mother liquor was of a slightly yellowish white color. After drying in vacuum over sulfuric acid it was decidedly hygroscopic and on exposure to the air it became sticky. Qualitative analysis showed that it contained phosphorus and nitrogen but no sulfur. On drying at the temperature of boiling chloroform in vacuum over phosphorus pentoxide it lost only 0.93 per cent in weight and the weight remained constant on further drying at 78°C. On analyzing it for phosphorus and nitrogen the following results were obtained.

Found. P 1.74, N 1.53 per cent.
Ratio N:P = 1.95:1.

The N:P ratio is nearly as 2:1 and the substance is, therefore, probably a diaminomonophosphatide.

Lack of time and material has prevented a complete examination of the phosphatides of corn pollen. The results obtained so far indicate that at least two phosphatides are present. We hope to prepare more material and will report on a more complete investigation of the corn pollen phosphatides in a later publication.

Examination of "Substance A."

It was mentioned previously that a small quantity of a colorless crystalline substance separated when the absolute alcoholic extract of pollen was allowed to stand over night. The crystals were filtered off, washed in a little absolute alcohol, and dried in vacuum over sulfuric acid. It weighed 0.2 gm. The substance was insoluble in water and very slightly soluble in cold alcohol, but readily soluble in ether, chloroform, and in hot alcohol.

The substance was twice recrystallized from boiling absolute alcohol from which it separated on cooling in small transparent plates. The dry crystals were snow-white in color and they exhibited a fatty feeling to the touch. It gave in somewhat modified form the Salkowski and Liebermann reaction of cholesterol or phytosterol. The melting point, however, was sharp at 88–89°C. (uncorrected). The small quantity of this substance prevented its identification but the crystal form and melting point correspond to those of myricyl alcohol.

Examination of "Substance B."

This substance separated in crystalline form on concentrating the absolute alcoholic extract of pollen. The crystals were filtered off and washed in alcohol. The substance was readily soluble in water and it crystallized again on adding alcohol to the aqueous solution. Through an accident the larger part of the material was lost, but from the small quantity which was saved we obtained after recrystallizing four times 0.75 gm. of beautiful colorless needle-shaped crystals. It gave the reaction of Scherer and melted at 221°C. (uncorrected), thus showing that the substance was pure inosite.

Quantitative Determination of Inosite in Corn Pollen.

60 gm. of the pollen were digested in 200 cc. of water with frequent shaking for 3 hours. It was then filtered through a layer of paper pulp and washed with water until 450 cc. of filtrate were obtained. The filtrate was yellow in color and it possessed a strong odor of pollen. It was evaporated to 50 cc. on the water bath, filtered, and the inosite was isolated by the method of Mayer (8). The pure colorless characteristic inosite crystals finally obtained weighed 0.5 gm. which corresponds to a yield of 0.83 per cent, but considering the inevitable losses during the isolation and purification it is very probable that corn pollen contains not less than 1 per cent of free inosite. The crystals gave the Scherer reaction and melted at 221°C. (uncorrected). We have no doubt whatever that the substance was pure inosite and the analysis was therefore omitted.

Extraction of the Pollen Residue with 70 Per Cent Alcohol.

The pollen residue which remained after extracting with ether and alcohol, as already described, was digested in 1 liter of 70 per cent alcohol at room temperature, with occasional stirring, for several days. It was then filtered on a Buchner funnel and washed with 70 per cent alcohol until about 1,300 cc. of filtrate were obtained. This solution was concentrated under reduced pressure to about one-half of its volume. It was then heated nearly to boiling and alcohol was added until the solution turned cloudy. After standing in the ice chest several days a considerable amount of a crystalline substance had separated.

The crystals were filtered off and washed in alcohol. They were dissolved in a little water, decolorized with animal charcoal, and again brought to crystallization by adding alcohol. After recrystallizing four times 1.4 gm. of colorless needle-shaped crystals were obtained. The crystal form was characteristic of inosite. The substance gave the reaction of Scherer and melted at 221°C. (uncorrected). Since the reactions and properties of this substance indicated that it was pure inosite the analysis was omitted.

After the above crystals of inosite had separated the mother liquor was concentrated to a thin syrup under reduced pressure at a temperature not exceeding 40°C. The syrup was taken up in a little water and precipitated with a solution of lead acetate. After settling, the precipitate was filtered on a Buchner funnel and washed with water.

The filtrate was freed from lead by hydrogen sulfide and the excess of hydrogen sulfide was removed by a current of air. The solution was then decolorized with animal charcoal and concentrated under reduced pressure. Sulfuric acid was added until the solution contained about 5 per cent of this acid. A concentrated solution of phosphotungstic acid was then added until no further precipitation occurred. After standing for several hours the precipitate was filtered and washed with 5 per cent sulfuric acid.

Unfortunately, the filtrate was lost through an accident which prevented an examination of it for amino-acids and soluble carbohydrates.

The phosphotungstic precipitate was rubbed up in a mortar with an excess of barium hydroxide, filtered, and the precipitate thoroughly washed with water. The filtrate was acidified slightly with sulfuric acid, filtered from barium sulfate, and concentrated under reduced pressure to about 400 cc. The solution was then made up to 500 cc. with water. Nitrogen was determined in this solution by the Kjeldahl method and it was found to contain 0.8470 gm. of nitrogen.

Through fractional precipitation with phosphotungstic acid we were able to separate the nitrogenous constituents into two principal fractions which were identified as choline and *l*-proline. The nitrogen recovered amounted to about 80 per cent, the balance being lost in the processes of separation and purification.

Separation of Choline.

An attempt was made to separate histone bases by the method of Kossel and Kutscher (9) as described by Steudel (10) but only traces of nitrogen were precipitated. After the solution was freed from barium and silver about 4 per cent of sulfuric acid and a very slight excess of phosphotungstic acid were added. The precipitate was filtered after standing over night and washed with 4 per cent sulfuric acid. The filtrate and washings were reserved for the preparation of *l*-proline.

The phosphotungstic precipitate was decomposed with barium hydroxide, the excess of barium removed with carbon dioxide, and the filtrate made up to 500 cc. This solution was found to contain 0.2327 gm. of nitrogen determined by the Kjeldahl method. This would correspond to 2.01 gm. or 0.34 per cent of choline in corn pollen. The solution was concentrated under reduced pressure, filtered from a small quantity of barium carbonate, and evaporated in a vacuum desiccator over sulfuric acid. The thick syrupy residue which remained was taken up in a little alcohol and to it was added an alcoholic solution of picric acid until the solution turned cloudy. On cooling the picrate separated in large needle-shaped crystals. These were filtered off and washed in a little alcohol. The filtrate and washings were concentrated and on cooling a further quantity of crystals were obtained which were added to the first lot. The picrate was recrystallized from a little hot water, filtered, washed with absolute alcohol, and dried in vacuum over sulfuric acid. The dry picrate weighed 2.67 gm. and it melted at 239–240°C. (uncorrected).

The picrate was suspended in about 50 cc. of water and acidified with hydrochloric acid and the picric acid was shaken out with ether. The aqueous solution was concentrated under reduced pressure and finally dried in vacuum over sulfuric acid until a mass of colorless crystals remained. The crystals were very hygroscopic and rapidly liquefied on exposure to the atmosphere. The xanthine and the Weidel reactions and the Kossel reaction for adenine were all negative.

The substance was taken up in about 20 cc. of absolute alcohol, and ether was added gradually until crystallization began. It was then placed in a freezing mixture for 1 hour. The crystals were

filtered, rapidly washed with absolute ether, and dried in vacuum over sulfuric acid. In this manner the substance was obtained in long fine colorless needles. It was very hygroscopic and on exposure to the air the crystals rapidly liquefied. The crystals did not melt or show any change when heated to 260°C.

The alcoholic solution of the substance gave an orange-colored precipitate with platinic chloride. The platinum double salt was prepared and after recrystallizing from hot water to which about 15 per cent of alcohol was added it was obtained in orange-colored octahedral crystals. The air-dried double salt melted with decomposition at 232°C. (uncorrected).

On analysis, after drying at 105°C. in vacuum over phosphorus pentoxide, values were obtained which agree with the theoretical composition of choline platinum chloride.

0.1742 gm. substance: 0.0548 gm. Pt.

$(C_5H_{14}NO\ Cl)_2\ Pt\ Cl_4$.	Calculated.	Pt 31.64 per cent.
	Found.	" 31.45 " "

Preparation of l-Proline.

After the choline fraction had been precipitated by phosphotungstic acid, the filtrate was freed from sulfuric and phosphotungstic acids by barium hydroxide. The filtered solution was evaporated under reduced pressure and the residue was taken up in a little hot water. A small quantity of barium carbonate was filtered off and the filtrate made up to 100 cc. with water. Nitrogen was determined by the Kjeldahl method and the solution was found to contain 0.4396 gm. of nitrogen.

The solution was acidified with hydrochloric acid and concentrated under reduced pressure and finally dried in vacuum over sulfuric acid. A thick syrup remained which on scratching with a glass rod immediately crystallized. The crystals were digested in a little absolute alcohol, filtered on a Buchner funnel, and washed with a little cold absolute alcohol and ether. After drying in the air the substance weighed 3 gm. and it was a nearly pure white crystalline powder. It was very soluble in water and in hot 95 per cent alcohol. It also dissolved readily in hot absolute alcohol and on cooling it separated in colorless prisms. The alcoholic solution gave no precipitate with platinic chloride or with picric acid. The substance showed an acid reaction on litmus and on ignition it left no residue.

The substance was twice recrystallized from hot absolute alcohol and was obtained in beautiful colorless prisms. When heated in a capillary tube it melted with gas formation at 206–207° (uncorrected). It contained nitrogen, but sulfur, phosphorus, and halogens were absent. Boiled with copper oxide it gave off a peculiar odor and a deep blue-colored solution resulted. This solution was filtered and evaporated when a deep blue-colored amorphous copper salt was obtained which was completely soluble in alcohol.

The substance was analyzed after drying at 105°C. in vacuum over phosphorus pentoxide. There was no loss in weight on drying.

0.1470 gm. substance: 0.1051 gm. H_2O and 0.2791 gm. CO_2.
0.1560 " " 16.9 cc. of nitrogen at 16° and 731 mm.
Found. C 51.78, H 8.00, N 12.30 per cent.
For $C_5H_9NO_2$ = 115.
Calculated. C 52.17, H 7.82, N 12.17 per cent.

In aqueous solution the substance had a specific rotation of −69.69°.

The properties and composition of this substance agree with those of *l*-proline although the rotation is lower than given by Fischer (11). The identity with proline was established by converting it into *dl*-proline as described by Fischer (11). The copper salt was obtained in the form of the characteristic deep blue-colored crystals.

The following results were obtained on analysis after drying at 105° in vacuum over phosphorus pentoxide.

For $(C_5H_8NO_2)_2Cu + 2H_2O$ = 327.5.	Calculated.	H_2O 10.99 per cent.
	Found.	" 10.84 " "
For $(C_5H_8NO_2)_2Cu$ = 291.5.	Calculated.	Cu 21.78 per cent.
	Found.	" 21.73 " "

Some of the *l*-proline was treated with phenylisocyanate in alkaline solution and the resulting compound was converted into the anhydride or hydantoin by heating with 4 per cent hydrochloric acid. After recrystallizing four times from aqueous alcohol, the reaction product was obtained in the form of fine colorless needles which melted at 143°C. (uncorrected). Proline hydantoin melts at 143°C.

Corn pollen contains a relatively large amount of proline. The solution from which the proline was prepared contained 0.4396 gm. of nitrogen which corresponds to 3.6 gm. or 0.6 per cent of free *l*-proline in the pollen.

Phosphorus in the Extracted Corn Pollen.

The pollen residue after extracting with ether, absolute alcohol, and 70 per cent alcohol was analyzed for phosphorus. Total phosphorus was determined after destroying the organic matter by the Neumann method. The total soluble and the inorganic phosphorus were determined in the extracts obtained on digesting the pollen residue during 5 hours in 1 per cent hydrochloric acid. The value for the organic phosphorus soluble in 1 per cent hydrochloric acid was obtained by subtracting the inorganic from the total soluble phosphorus. The results are given in Table V.

TABLE V.

Forms of Phosphorus in Corn Pollen after Extracting It with Ether, Absolute Alcohol, and 70 Per Cent Alcohol.

Total phosphorus.	Total soluble phosphorus.	Inorganic phosphorus.	Organic phosphorus.
per cent	*per cent*	*per cent*	*per cent*
0.43	0.29	0.22	0.07

The organic phosphorus in Table V corresponds to the phytin phosphorus as determined in other plant material. Judging by the very small difference between the total soluble and the inorganic phosphorus in pollen it appears rather doubtful if phytin or inosite hexaphosphoric acid is present in this material.

Nitrogen in the Extracted Corn Pollen.

The pollen residue remaining after extracting with ether, absolute alcohol, and 70 per cent alcohol was analyzed for nitrogen. The material was extracted with the solvents mentioned in the table and the results obtained are given briefly in Table VI.

In earlier investigations the presence of malic acid has been reported in the pollen of *Phœnix dactylifera* by Fourcroy (12) and in the pollen of *Typha latifolia* by Braconnot (13) and Kresling (14) found tartaric and malic acid in the pollen of *Pinus sylvestris*.

In the case of corn pollen we were unable to obtain any evidence of the presence of any of these acids. The only acid which we could find in aqueous extracts of corn pollen was phosphoric acid in the form of calcium phosphate.

The soluble constituents which were isolated from corn pollen and identified are given in Table VII. The percentages are calculated to the dry pollen.

TABLE VI.

Nitrogen in the Extracted Corn Pollen.

Total nitrogen.	Nitrogen soluble in 1 per cent NaOH. Digested on water bath for 24 hours.	Nitrogen soluble in 1 per cent hydrochloric acid. Digested on water bath for 24 hours.	Nitrogen soluble in 5 per cent NaCl. Digested at 25°C. for 24 hours.	Nitrogen soluble in distilled water. Digested at 25°C. for 24 hours.
per cent	*per cent*	*per cent*	*per cent*	*per cent*
5.40	4.93	3.20	0.40	0.20

TABLE VII.

Soluble Identified Constituents Occurring in Corn Pollen.

Substance.	Per cent.
Amorphous phosphatide	1.94
Crystalline phosphatide	0.67
Inosite	0.83
Choline	0.34
l-Proline	0.60
Myricyl alcohol	Trace.

Some attempts were made towards the isolation of proteins, nucleic acid, and certain carbohydrates from pollen but these experiments are still incomplete. We hope to present a fuller report on the above constituents in a later publication.

SUMMARY.

The approximate composition of the pollen from three varieties of corn has been determined and the results indicate a difference in the composition of the pollen from different varieties of corn. A complete analysis of the ash of the pollen from one variety of corn is given.

Evidence is presented which indicates the presence of at least two phosphatides in corn pollen. One was an amorphous substance which also contained sulfur but the other was a crystalline phosphatide.

Relatively large quantities of free inosite, *l*-proline, and choline occur in corn pollen.

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THE RÔLE OF CEPHALIN IN BLOOD COAGULATION.

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The process of blood clotting is very complex. As it occurs normally, it is the result of many factors not well understood. The progress made in recent years was due to the fact that ways were found to separate the many factors into groups, some of which control definite phases of the complex process of blood clotting. The phase best understood is that of the conversion of fibrinogen into fibrin. Four substances take part in this reaction. One is the substrate, and the other three combine to bring about the transformation of the substrate from a soluble into an insoluble state. Alexander Schmidt understood the process correctly. More recent workers brought out many of the details of the process. The most recent contributions were made by Bordet and his collaborators, and by Howell and his coworkers.

The terms applied to each of the factors differed with the individual author. Since the blood clotting experiments here reported were carried out by a worker of Bordet's school, the nomenclature employed in this publication is of that school. In terms of that school the process of fibrin formation is expressed by the following diagram.

$$\left.\begin{array}{l}\text{Plasma at the}\\ \text{moment of coagulation}\\ \quad\text{or}\\ \text{serum after coagulation}\end{array}\right\}\text{serozyme}$$

$$\text{in presence of Ca ions} \longrightarrow \text{thrombin}$$

$$\left.\begin{array}{l}\text{Platelets}\\ \quad\text{or}\\ \text{tissue extracts}\\ \quad\text{or}\\ \text{certain lipoids}\end{array}\right\}\text{cytozyme} \qquad + $$

$$\text{Fibrinogen} = \text{fibrin}$$

Howell accepts the interplay of all four of the substances in the process of blood clotting but holds a different view on the rôle of cytozyme, which, according to Howell, plays no part in the actual transformation of fibrinogen into fibrin. Howell also disagrees with other workers in his view on the chemical nature of cytozyme. Alexander Schmidt, Wooldridge, Bordet, Delange and others regarded the substance as lecithin. This opinion was based on the thermostability of the substance and on its solubility in alcohol. Howell on the contrary came to the conclusion that the active substance was another phosphatide; namely, cephalin. Howell and his coworkers have also made an attempt to associate the activity of the phosphatide with a definite peculiarity of its chemical structure. At the time of the work of Howell it was generally accepted that the unsaturated acid entering into the structure of cephalin differed from that of lecithin. From cephalin, linolic acid was isolated and from lecithin, oleic acid. According to Howell and McLean the higher unsaturation of the fatty acid is the factor which lends to cephalin its property of being an agent in the formation of thrombin.

The present communication is a mere note dealing not with the entire problem of fibrin formation but only with the chemical nature of cytozyme. Is cytozyme lecithin or cephalin? Since the work of Howell and his coworkers, the knowledge of the chemical structure of phosphatides has made considerable progress. In the light of this progress the conclusions regarding the chemical nature of cytozyme required reinvestigation.

The recent work on lecithin has brought out the fact that there exist forms of this substance which contain in their molecule a fatty acid of still higher unsaturation than the one previously isolated from cephalin. In the light of the theory of Howell and McLean, one might have expected the new form of lecithin to play the same part as cephalin in fibrin formation.

Furthermore, recent work on cephalin has brought out the fact that the material handled by the older writers under the name of lecithin was in reality a complex mixture and not a uniform substance. The components of this mixture were found to be identical in character with those of another complex mixture described by previous writers under the name of cuorin, or heparphosphatide. Cuorin and the cephalin of the older writers

differed one from another in the proportions of some of their components. Both substances were found to consist of true cephalin, true lecithin, and of the same substances in a state of partial decomposition. The character of the decomposition products varied from sample to sample. Yet, Howell and his coworkers observed that cephalin and cuorin acted in the process of blood coagulation antagonistically to one another.

On the other hand, a substance was recently prepared which was free from the decomposition product of lecithin and of cephalin and which contained 75 per cent of undecomposed cephalin and 25 per cent of undecomposed lecithin. Whether or not the substance contained impurities undetectable by the present methods of analysis, cannot be stated.

It is self-evident that it became important to compare the cytozymic function of the three substances; namely, of ordinary lecithin, of lecithin which contained the fatty acid of a high degree of unsaturation, and of the new cephalin material. In a way also the present materials were mixtures. Ordinary lecithin contains a small proportion of the new form. The new form still contained a very small proportion of the older type. The cephalin contained a small proportion of lecithin. Yet even such material was sufficient to bring out the fact that lecithin, regardless of its form and of its origin, possesses no cytozymic action. On the other hand, material containing 75 per cent of undecomposed cephalin and 25 per cent of lecithin possesses unusually high cytozymic action. It is still active in a concentration of $5 (10^{-7})$.

The coagulation experiments were carried out by Dr. Gratia who followed the routine customary in Bordet's school. The plan and the details of the experiments follow.

EXPERIMENTAL PART.

Oxalated plasma from which most of the platelets have been removed by centrifugation contains only a small amount of cytozyme and consequently clots very slowly when recalcified, but clots quickly if some cytozyme is given back in form either of platelet suspension, tissue juice, or lipoidic tissue extract. This offers means of testing the cytozymic properties of a given lipoid by measuring the accelerating influence of the lipoid on the coagulation of a plasma almost free from platelets.

When an oxalated plasma has been strongly centrifugalized and then recalcified, the few remaining platelets contain just enough cytozyme to react with but a small part of the serozyme and thus yield only a small amount of thrombin. The plasma clots slowly and a great excess of unutilized serozyme is found in the serum after coagulation. Such a serum is rich in serozyme and is an excellent reagent to test the cytozymic properties of a given lipoid. If cytozyme even in very small amount is added to this serum, an active production of thrombin immediately results and this mixture is able to clot an equal volume of fibrinogen or oxalated plasma in a few minutes. This is the serozyme-cytozyme reaction of Bordet and Delange.

In our researches we have submitted our different lipoids to both tests. The materials used were prepared as follows:

Preparation of the Reagents.

1. Lipoidic Emulsions.—1 per cent emulsions of our three lipoids were made in saline solution. As a control a similar 1 per cent emulsion was made with lipoidic extract of tissue which was known to possess strong cytozymic properties. When necessary, higher dilutions of these suspensions were made in the course of the experiments.

2. Oxalated Plasma Free from Platelets.—A rabbit was carefully bled from the carotids with a paraffined cannula. Precautions were taken to avoid the contact of the blood with tissue juice and 9 parts of blood were received in 1 part of a 1 per cent solution of sodium oxalate in saline solution, and thoroughly mixed. This 1 per cent oxalated blood was centrifugalized at high speed during 1 hour and the clear supernatant plasma removed from the cells with a pipette. For use in the experiments 1 part of this oxalated plasma (O. P.) was recalcified with 4 parts of a 0.35 per cent solution of calcium chloride in saline solution (Ca).

3. Serum Rich in Serozyme.—A few cc. of oxalated plasma were recalcified as above described. When coagulation began, the recalcified plasma was defribinated with a glass rod. The serum obtained was kept at room temperature until the next day. As thrombin is very labile, the small amount of thrombin left after this very slow coagulation disappears quickly and the next day the serum containing nothing but a large excess of serozyme is ready for use.

4. Fibrinogen.—Instead of the so called pure solution of fibrinogen, "dioxalated plasma" (F) was used as a test for thrombin. This very convenient reagent was prepared according to the technique of Bordet and Delange; *i.e.*, 1 part of 1 per cent oxalated plasma was diluted with 4 parts of a 2 per cent solution of sodium oxalate in saline solution.

A. Egg Lecithin.

Experiment I.

0.25 cc. O.P.	+ 1 drop	saline solution	+ 7 cc. Ca	= 110′
0.25 “ “	+ 1 “	egg lecithin	+ 7 “ “	= 90′
0.25 “ “	+ 1 “	cytozyme	+ 7 “ “	= 20′

Egg lecithin exerts only a slight accelerating influence on the coagulation of recalcified oxalated plasma.

Experiment II.

0.25 cc. serozyme	+ 1 drop	saline solution	..5′...	+ 0.25 cc. F = ∞
0.25 “ “	+ 1 “	egg lecithin	..5′...	+ 0.25 “ “ = still fluid after 5 hrs.; soft clot after 24 hrs.
0.25 “ “	+ 1 “	cytozyme	...5′...	+ 0.25 cc. F = 2′

Whereas after 5′ a mixture of serum rich in serozyme together with cytozyme contains a sufficient quantity of thrombin to clot an equal volume of oxalated plasma in 2′, a similar mixture of serozyme with lecithin contains only a practically negligible amount of thrombin that yields hardly a soft clot after 24 hours.

The 1 per cent emulsion of lecithin is rather viscous. Dilutions of the lecithin as well as of the cytozyme emulsions were made, 1/10, 1/100, 1/1,000, and compared.

Experiment III.

0.25 cc. serozyme	+ 1 drop	cytozyme 1/10	...5′...	+ 0.25 cc.	F = 2′
0.25 “ “	+ 1 “	lecithin 1/10	...5′...	+ 0.25 “	“ = ∞
0.25 “ “	+ 1 “	cytozyme 1/100	...5′...	+ 0.25 “	“ = 8′
0.25 “ “	+ 1 “	lecithin 1/100	..5′...	+ 0.25 “	“ = ∞
0.25 “ “	+ 1 “	cytozyme 1/1,000	..5′...	+ 0.25 “	“ = 25′
0.25 “ “	+ 1 “	lecithin 1/1,000	..5′...	+ 0.25 “	“ = ∞

Egg lecithin is thus inactive at higher dilutions. In the following series the tests were allowed to react at longer intervals.

Experiment IV.

0.25 cc. serozyme + 1 drop lecithin ... 5'... + 0.25 cc. F = ∞
0.25 " " + 1 " " ...10'... + 0.25 " " = ∞
0.25 " " + 1 " " ...15'... + 0.25 " " = ∞
0.25 " " + 1 " " ...20'... + 0.25 " " = ∞
0.25 " " + 1 " " ...45'... + 0.25 " " = ∞

The results again were negative. The following series aims to establish whether lecithin in any way affected the potency of the cytozyme.

Experiment V.

0.25 cc. serozyme + 1 drop cytozyme + 1 drop saline solution ..5'.. + 0.25 cc. F = 2'

0.25 cc. serozyme + 1 drop cytozyme + 1 drop lecithin ....5'.... + 0.25 cc. F = 2'

0.25 cc. serozyme + 1 drop cytozyme + 3 drops lecithin ....5'.... + 0.25 cc. F = 2'

0.25 cc. serozyme + 1 drop cytozyme 1/10 + 1 drop saline solution ..5'.. + 0.25 cc. F = 2'

0.25 cc. serozyme + 1 drop cytozyme 1/10 + 1 drop lecithin ..5'.. + 0.25 cc. F = 2'

0.25 cc. serozyme + 1 drop cytozyme 1/100 + 1 drop saline solution ..5'.. + 0.25 cc. F = 10'

0.25 cc. serozyme + 1 drop cytozyme 1/100 + 1 drop lecithin ..5'.. + 0.25 cc. F = 10'

The results show that there was no appreciable accelerating inhibiting influence of egg lecithin on the action of cytozyme.

Conclusion.—Egg lecithin has practically no cytozymic properties. The extremely small action observed in Experiments I and II must very likely be due to traces of the active substance still present as an impurity in the egg lecithin material.

B. Liver Lecithin.

Identical experiments were repeated with the liver lecithin with similar results. Thus the liver lecithin is not more active than egg lecithin.

C. Mixtures of Pure Cephalin and Pure Lecithin.

Experiment VI.

0.25 cc. O.P. + 1 drop saline solution + 1 cc. Ca = 50'
0.25 " " + 1 " cytozyme + 1 " " = 11'
0.25 " " + 1 " cephalin + 1 " " = 10'

The mixture containing 65 per cent of pure cephalin has a marked accelerating effect on the coagulation of recalcified oxalated plasma.

Experiment VII.

0.25 cc. serozyme	+ 1 drop saline solution		...5'...	+ 0.25 cc.	F	= ∞
0.25 " "	+ 1 " cytozyme		...5'...	+ 0 25 "	"	= 2'
0.25 " "	+ 1 " cephalin		...5'...	+ 0.25 "	"	= 1'
0.25 " "	+ 1 " "	1/10	...5'...	+ 0 25 "	"	= 1'
0.25 " "	+ 1 " "	1/100	...5'...	+ 0.25 "	"	= 3'
0.25 " "	+ 1 " "	1/1,000	...5'...	+ 0.25 "	"	= 25'
0.25 " "	+ 1 " "	1/10,000	...5'...	+ 0.25 "	"	= ∞

1 drop of 7 per cent cephalin emulsion, even diluted 1:1,000, is still able to give a positive result. The calculated amount of material contained in this drop is about 1/20,000 of a mg. This will give an idea of the extraordinary cytozymic activity of the mixture of cephalin and lecithin.

THE HEAT OF REACTION OF OXYGEN WITH HEMOGLOBIN.

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A quantitative understanding of the physical chemistry of oxyhemoglobin requires a knowledge of its heat of formation from oxygen and hemoglobin. With the help of this quantity there may be deduced certain thermodynamic properties of the compound, provided further complexities do not mask the application of simple principles. The present paper gives the results of new measurements of the heat developed when oxygen combines with dissolved hemoglobin, and serves to demonstrate the presence of such complexities. The method used in the thermochemical study of gas-liquid reactions is described, and values obtained by it for the following other reactions are included in the data:

Carbon dioxide + water.
" " + solutions of alkalies.
Oxygen + pyrogallol solutions.
Carbon monoxide + hemoglobin solutions.
" dioxide + blood.

Apparatus.

Gas at a standard temperature and pressure was bubbled through the reacting solution contained in an insulated calorimeter immersed in a thermostat. Temperature changes were read upon a Beckmann's thermometer.

The apparatus is represented in Fig. 1. A silvered Dewar flask of about 230 cc. capacity served as the calorimeter ves-

* The data contained in this paper are taken from a thesis presented by Edward F. Adolph to the faculty of Harvard University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

sel. It was 3 cm. in diameter and 30 cm. deep, so that a long exposure was given to gas bubbling upward. It was closed by a rubber stopper, through which passed the thermometer, an inlet tube reaching to the bottom and ending in a finely perforated bulb, and an outlet tube opening to the air.

The Beckmann's thermometer was graduated in hundredths of a degree Centigrade, and was read to 0.001°. It was calibrated for the range 20.5 to 22.5°C. in an adiabatic calorimeter by comparison with a platinum resistance thermometer in the laboratory of Professor T. W. Richards. We are indebted to Mr. Bridgeman for aid in this calibration. At nine points of comparison the largest correction found for 0.25° was $\pm 0.003°$, and over a range of 2.0° the correction was $+0.0056°$.

The thermostat held 50 liters of water which was maintained at 22°C. $\pm$ 0.02°. A toluene regulator with a mercury-platinum contact was connected to a relay and under its control a set of lamps supplied heat intermittently. A separate current ran a motor which turned a stirrer.

The gases used were oxygen, hydrogen, carbon dioxide, and carbon monoxide. The oxygen supply was drawn from a pressure tank, and contained no carbon dioxide or carbon monoxide. Before use it passed through two wash bottles which contained sodium hydroxide solution. Carbon dioxide also was obtained from a tank. It contained less than 0.5 per cent of air, and was washed only in water. Hydrogen was generated in a small Kipp generator from zinc and hydrochloric acid. After the generator had started, there was no access for air until the zinc was replenished. The gas passed through two wash bottles containing strong solutions of pyrogallol and sodium hydroxide. Carbon monoxide was generated by dropping formic acid into sulfuric acid. It was washed in three bottles of pyrogallol and sodium hydroxide solution.

With each gas a reservoir bottle of 10 liters capacity was filled. A constant flow of gas from the reservoir toward the calorimeter was secured by running water into the reservoir from a second bottle raised above it. Into the second bottle water was run from a faucet at such a rate that the levels in both bottles rose equally, and thus the hydrostatic pressure was constant throughout the flow of gas. To refill the reservoir the water was poured

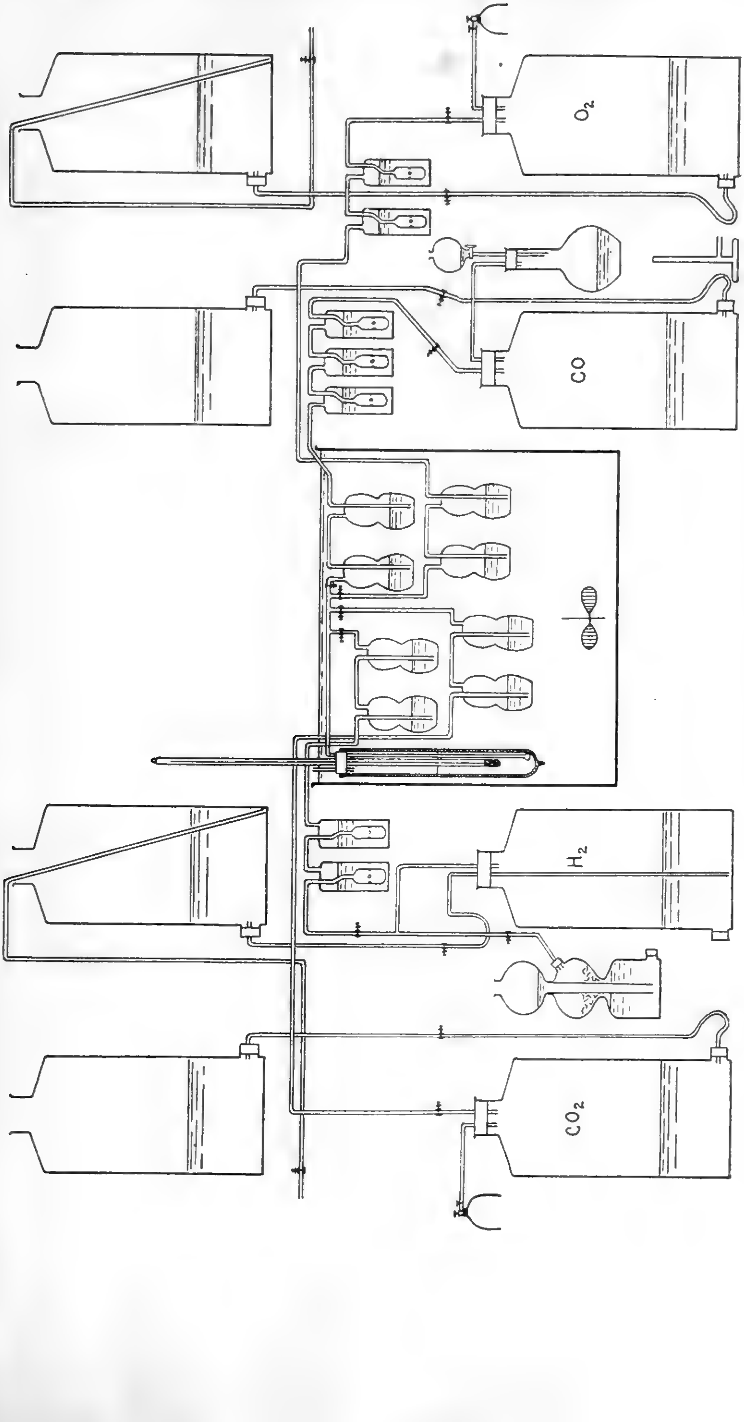

FIG. 1. Diagram of thermochemical apparatus.

from the upper bottle; and this bottle was lowered to receive the water displaced by gas entering the reservoir.

During an experiment the gas left its reservoir at a rate of 400 cc. per minute. It passed through its special wash bottles, and then through a pair of wash bottles immersed in the thermostat. Each gas had a separate pair of these, all containing 0.9 per cent NaCl solution. In them the gas attained the temperature of the bath (22°C.), and was completely saturated with moisture at the vapor tension of this solution. The four gas systems united at the entrance to the calorimeter, and by the manipulation of the necessary stop-cocks one gas flow could be substituted for another in about 30 seconds. The rapid bubbling of gas served to stir the reacting solution in the Dewar flask.

The tube leading out from the calorimeter maintained atmospheric pressure within, and served for sampling the solution by the mere insertion of a pipette.

The foaming of solutions such as hemoglobin and blood was sufficiently inhibited by the addition of a few drops of octyl alcohol.

EXPERIMENTAL PROCEDURE.

A known amount of solution (usually 100 cc.), at about 22°C., was run into the calorimeter. A non-reacting gas was made to flow through it, and thermometer readings were recorded every 60 seconds. The non-reacting gas exhibited the rate of temperature change due to all constant influences, of which the chief was the small difference in temperature between calorimeter and thermostat. This was the fore period, usually lasting 5 minutes. Then the reacting gas was run in and the reaction carried to completion, which required 8 to 12 minutes in most instances. A final period was obtained by continuing the passage of the reactive gas after all chemical change had occurred.

At completion of an experiment, the thermometer readings were plotted against time. A line through the readings of the final period was extrapolated back to the time when the reaction began (Fig. 4). The temperature difference between the extrapolated and the initial readings represents approximately that due to chemical action. For inorganic reactions a greater accuracy was gained by taking the cooling curve for the first half of the reaction period from the readings of the fore period, and only

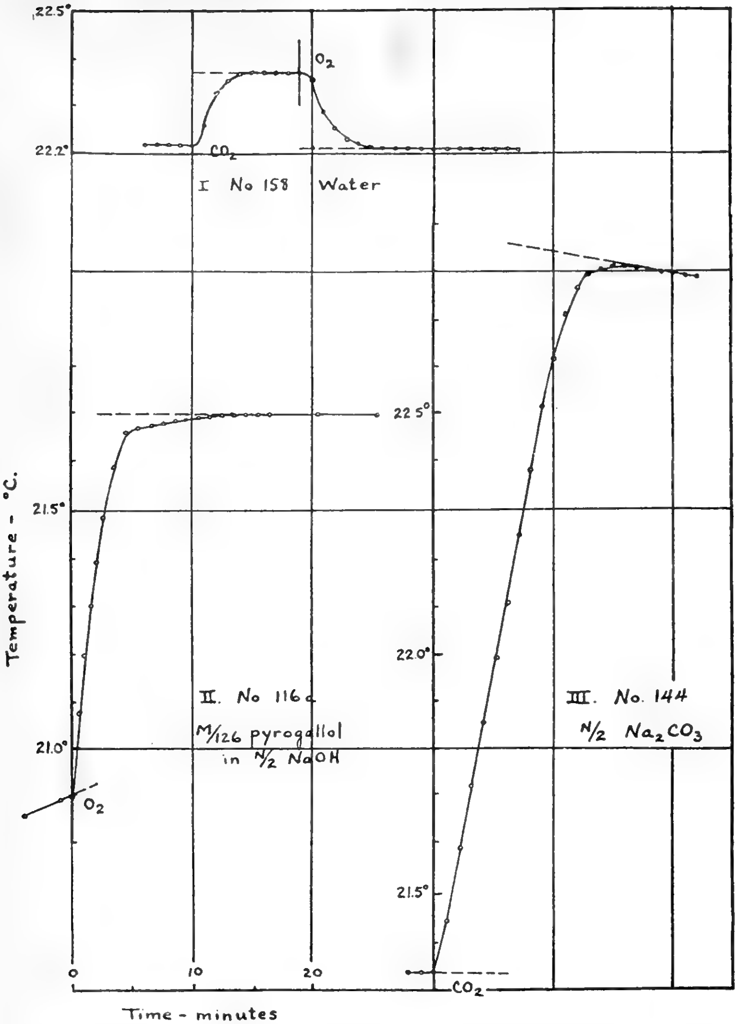

FIG. 2. Heats evolved in dissolving a gram molecule of gas in water at various temperatures; calculated from the solubility data.

the last half from those of the final period (Fig. 2). For rises of temperature of more than 0.5° the method of Pfaundler (1) was used to calculate the cooling correction.

Before calculating the heat production it was necessary to know the heat capacities. The capacity of the calorimeter was roughly determined by three methods: causing solutions of hydrochloric acid and of sodium hydroxide to react in it; introducing water at known temperatures; and calculating the heat capacity of the glass of which it was made. When containing 100 cc. of solution with the thermometer and inlet tube in place, the heat capacity of the calorimeter was 14 calories per degree Centigrade.

The heat capacity of the solutions was calculated. For inorganic solutions the available data (2) were plotted, and the capacity corresponding to the concentrations read off from the curve. For purified hemoglobin solutions a calculation was made on the assumption that the protein has a specific heat of 0.4 calorie per degree Centigrade per gram when dried. For defibrinated blood, concentrated corpuscles, and serum, the values of Hillersohn (3) and of Bordier (4) were used.

Experiments with Inorganic Solutions.

Heats of Solution of Gases.—The rise of temperature when carbon dioxide passes into water, and its fall when oxygen displaces the carbon dioxide from saturated solution, was measured. Assuming that the solubility of carbon dioxide at 22°C. and 760 mm. pressure is 83 volumes per cent (5) the heat of solution was calculated (Table I). The average result of eleven determinations at 22°C. is +4,690 calories per gram molecule of carbon dioxide (standard deviation 2.9 per cent). The chief error in these determinations is the assumed solubility for carbon dioxide. Thomsen (6) found +5,882 calories developed at 19°C., Berthelot (7) found + 5,600 calories at 15°C.

Owing to the small solubility in water of oxygen, nitrogen, hydrogen, and carbon monoxide, it was not possible to measure their heats of solution. The "isochore" of van't Hoff (8) furnishes a method of calculating them, however, for the variation of their solubility with temperature is known from the data of Bohr and Bock (5) and of Winkler (9). Van't Hoff showed that if K_1 and K_2 are the equilibrium (solubility) constants at two absolute temperatures T_1 and T_2, R is the gas constant in heat

TABLE I.

Heat of Solution of CO_2 in Water.

(1) No.	(2) Corrected rise in temperature.	(3) Heat developed per gram molecule CO_2.	(4) Average heat of solution per gram molecule CO_2.
	°C.	*calories*	*calories*
157	0.163	4,940	
158	0.153	4,630	
196	0.154	4,670	
199	0.153	4,630	
222	0.150	4,540	
260	0.150	4,540	+4,690
260a	0.153	4,630	
147	−0.161	4,880	
157a	−0.158	4,780	
158a	−0.160	4,840	
199a	−0.150	4,540	

Formula for calculation: $(3) = \dfrac{(2) \times 112 \times 22{,}400}{0.829 \times 100}$

units, and Q is the total energy change expressed as heat evolved, then

$$Q = \frac{R\,T_1T_2}{T_1 - T_2} \times \log_e \frac{K_1}{K_2}\ .$$

Values of Q, calculated for 5° intervals of temperature, are plotted in Fig. 3 for each of five gases. Reading from these curves, the values at 22°C. turn out to be:

O_2 +3,100 calories per gram molecule.
N_2 +3,000 " " " "
H_2 +1,200 " " " "
CO +2,800 " " " "
CO_2 +5,000 " " " "

The calculated value for carbon dioxide agrees well with the experimental value. None of the gases is appreciably ionized in solution.

Heats of Reaction of CO_2 with Alkalies in Solution.—In these reactions the temperature rise could be made sufficient to yield very accurate results. These are given in Table II. It will be seen that the average value for the reaction of the dissolved gas

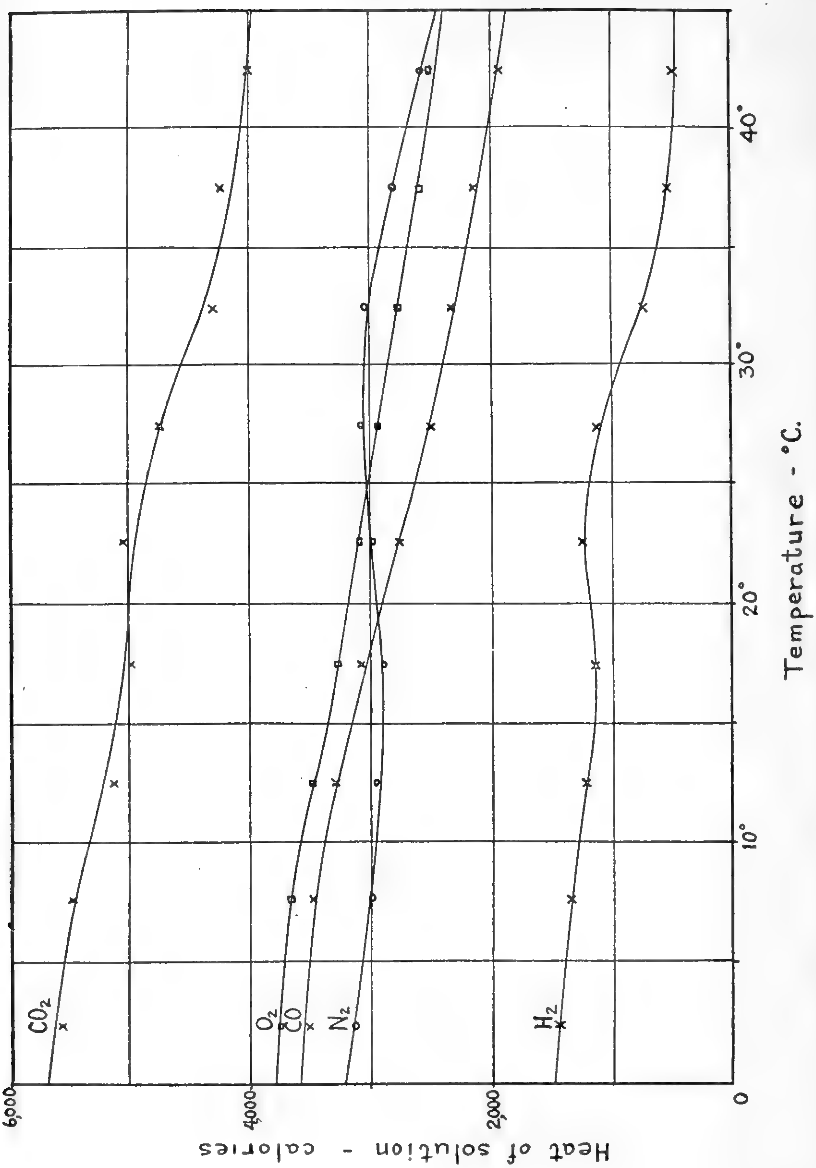

FIG. 3. Temperature changes in the course of three thermochemical measurements.

with sodium hydroxide or potassium hydroxide solutions is +10, 690 calories per gram molecule at 22°C. The standard deviation among ten determinations is 1.0 per cent. Thomsen (6) obtained the value +11,016 calories at 18°C., and Berthelot (7)

TABLE II.

Heat of Reaction of CO_2 with Alkalies.

(1) No.	(2) Solution.	(3) Solution.	(4) Temperature rise corrected for cooling.	(5) Heat capacity per degree.	(6) Heat due to CO_2 dissolved in the solution.	(7) Heat developed per gram molecule of combined CO_2.	(8) Average heat of reaction.
		cc.	*°C.*	*calories*	*calories*	*calories*	*colories*
119	0.298 M NaOH.......	50	4.282	56	8.3	15,480	
120	0.298 M "	50	4.209	56	8.3	15,220	
121	0.298 M "	50	4.166	56	8.3	15,060	
122	0.298 M "	50	4.183	56	8.3	15,110	
123	0.298 M "	50	4.211	56	8.3	15,240	
124	0.298 M "	50	4.214	56	8.3	15,250	+10,600
132	0.149 M "	100	2.216	111	16.8	15,270	
136	0.149 M "	100	2.225	111	16.8	15,350	
138	0.181 M KOH........	100	2.706	111	16.8	15,600	
139	0.181 M "	100	2.665	111	16.8	15,350	
142	0.250 M Na_2CO_3	100	1.513	109	16.2	5,950	
143	0.250 M "	100	1.541	109	16.2	6,070	+1,250
144	0.250 M "	100	1.481	109	16.2	5,810	
243	0.067 M Na_2HPO_4....	100	0.323	111	17.0	2,830	
257	0.067 M "	100	0.298	111	17.0	2,420	−1,900
261	0.067 M "	100	0.328	111	17.0	2,910	
258	0.067 M KH_2PO_4.....	100	0.147	111	17.0	0	0

Formula for calculation: $(7) = \dfrac{\{[(4) \times (5)] - (6)\} \times 1,000}{(2) \times (3)}$

+11,100 calories at 18°C., for the reaction of solutions of carbonic acid with solutions of sodium hydroxide.

The heat of neutralization of strong acids and strong bases at 22°C. is +13,600 calories. Using the above experimental results, the heat of ionization of carbonic acid to bicarbonate is

−2,910 calories. Thomsen (6) found it to be −2,800 calories at 18°C., and Kendall (10) calculated by the isochore that it would be −2,830 calories at 22°C.

Heat of Oxidation of Pyrogallol Solutions.—The chemistry of the reaction of oxygen with pyrogallol is not known, and varies with dilution and other factors. Berthelot (11), however, has shown that each molecule of sodium pyrogallate absorbs 3 atoms of oxygen under most conditions. No inactive gas was used in the fore period, but oxygen was bubbled through a solution of 0.5 M sodium hydroxide, and after 5 minutes a weighed amount of crystalline pyrogallol was added (Table III).

TABLE III.

Heat of Oxidation of Pyrogallol.

(1) No.	(2) Pyrogallol.	(3) Solution.	(4) Temperature rise.	(5) Heat capacity.	(6) Heat developed per gram molecule pyrogallol.	(7) Average heat of reaction.
	gm.	*cc.*	*°C.*	$\frac{calories}{degree}$	*calories*	*calories*
114	0.050	100	0.288	112	79,500	+65,000
114a	0.100	100	0.825	112	113,700	
115	0.050	50	0.712	57	99,800	
116	0.050	50	0.810	57	113,500	+95,900
116a	0.050	50	0.783	57	109,700	
117	0.050	50	0.824	57	115,300	

$$\text{Formula for calculation:}\quad (6) = \frac{(4) \times (5) \times 126}{(2)}$$

The heat of solution of pyrogallol, and its heat of complete neutralization, were measured by Berthelot (12) and by de Forcrand (13). Averages of their values (−3,590 and +13,440) were subtracted and a further correction was made for the heat of solution of 3 gram atoms of oxygen, to give the final result of +95,900 calories.

The three varieties of gas reactions studied above indicate the reliability of the method, and demonstrate that it can attain an accuracy of about 1 per cent. Such an accuracy was deemed more than sufficient for the study of the oxygenation of hemoglobin in solution.

Experiments with Hemoglobin Solutions and Blood.

Heat of Reaction of Oxygen and Carbon Monoxide with Hemoglobin Solutions.—The quantitative results depend upon the fact that 1 molecule of oxygen or of carbon monoxide combines chemically with that amount of hemoglobin which contains 1 atom of iron. The molecular weight of hemoglobin has been assumed to be 16,700. Corrections have been made for the heats of solution of the gases, both in measuring heats of reaction and in comparing the values of the equilibrium constant for oxyhemoglobin (K) and for carbon monoxide hemoglobin (k) at various temperatures.

Several investigators have measured the heats of reaction of oxygen and of carbon monoxide with hemoglobin in blood. Without attempting a critique of their methods, a summary of their results is presented in Table IV. In addition to the experiments of four authors included in the table, the work of Camis (18) and of Meyerhof (19) should be mentioned. Camis obtained a negative heat of formation of oxyhemoglobin, though his results have usually been interpreted otherwise. Meyerhof found roughly that when fresh blood was oxygenated, the heat produced just balanced that absorbed due to carbon dioxide carried out of solution.

To avoid as many complications as possible, it was decided to work chiefly with purified hemoglobin solutions. Washed corpuscles from defibrinated beef blood were dialyzed by a method which has been already described (20). Before use the solution of dialyzed hemoglobin was boiled at 40°C. *in vacuo* to remove all gases. It was cooled to 22°C., measured into the calorimeter without exposure to air, and subjected to a stream of hydrogen. After a few minutes the flow of gas was stopped while a sample of solution for analysis was pipetted from the calorimeter. At the end of the passage of the reacting gas a second sample was taken for analysis.

All oxygen and carbon monoxide analyses were performed by the method of Van Slyke (21). In every case a correction was made for dissolved gases. There is some evidence that the quantities thus measured may be too large (22). Carbon dioxide was measured with the same apparatus, using Van Slyke's technique (23), except that lactic acid replaced sulfuric acid as the reagent

TABLE IV.

Previous Measurements of Heat of Reaction.

(1) Author.	(2) Hemoglobin preparation.	(3) Temperature.	(4) Temperature rise.	(5) Gas absorbed.	(6) Heat developed per gm. Hb.	(7) Heat developed per gram molecule Hb.	(8) Heat of solution calculated.	(9) Average molal heat of reaction.
Oxygen + hemoglobin.								
		°C.	°C.	*vol. per cent*	*calories*	*calories*	*calories*	*calories*
Berthelot (14).	Defibrinated sheep blood.	9	0.115	20.2	0.895	14,960	3,500	11,640
			0.108	18.3	0.918	15,320		
Torup (15).	Crystalline horse Hb.	17	0.050	4.8	0.754	12,600	–	11,300
			0.042	3.7	0.658	11,000		
			0.041	3.7	0.623	10,400		
			0.046	4.6	0.678	11,300		
Barcroft and Hill (16).	Crystalline dialyzed dog Hb.	16	0.138	11.9	1.82	30,400	3,300	27,600
					1.98	33,070		
					1.75	29,230		
Du Bois-Reymond (17).	Defibrinated horse blood.	20	0.17	19	1.06	17,700	3,200	19,650
			0.16	21	1.09	18,200		
			0.32	34	1.21	20,200		
			0.11	11	1.33	22,200		
			0.24	23	1.39	23,200		
			0.34	26	1.73	28,900		
			0.30	23	1.77	29,600		
		43	0.08	10	0.97	16,200	2,400	13,800
	Crystalline horse Hb.	20	0.04	6.7	0.80	13,400	3,200	13,800
			0.12	16.2	0.99	165,00		
			0.02	2.3	1.14	19,000		
			0.02	2.3	1.14	19,000		
	Defibrinated horse blood. Only partially reduced.	20	0.04	5	1.05	17,500	3,200	15,100
			0.08	10	1.05	17,500		
			0.09	10	1.20	20,000		

TABLE IV—*Concluded.*

(1) Author.	(2) Hemoglobin preparation.	(3) Temperature.	(4) Temperature rise.	(5) Gas absorbed.	(6) Heat developed per gm. Hb.	(7) Heat developed per gram molecule Hb.	(8) Heat of solution calculated.	(9) Average molal heat of reaction.
			Carbon monoxide + hemoglobin.					
		°C.	*°C.*	*vol. per cent*	*calories*	*calories*	*calories*	*calories*
Berthelot (14).	Defibrinated sheep blood.	9	0.098		1.08	18,030	3,400	15,300
			0.125		1.16	19,300		
Du Bois-Reymond (17).	Defibrinated horse blood.	38	0.17	19	1.2	20,000	2,100	17,800
			0.23	33	0.9	15,000		
		20	0.19	22	1.2	20,000	2,900	
			0.20	17	1.6	26,700		
			0.09	10	1.2	20,000		

to avoid the precipitation of hemoglobin. Frequent analyses of evacuated samples show that the average amount of carbon dioxide present was less than 2 volumes per cent.

Any fall in temperature due to the expulsion of carbon dioxide was automatically corrected for in the graphical method of calculating the cooling (Fig. 4), since the fall is continued after the oxygenation is complete. Any heat production due to internal metabolism of blood, such as Meyerhof (19) measured, was also compensated by the correction. A third error similarly cared for was the evaporation of octyl alcohol. Evaporation of water was prevented by the complete saturation of the gases going into the calorimeter; even failing this it was likewise offset by the method of experimentation.

Reaction of Oxygen with Hemoglobin Solutions.—All the complete measurements with oxygen are given in Table V.

The average result for 35 measurements at 22°C. turned out to be +10,050 calories per gram molecule of oxygen, standard deviation ±2,320 calories (22 per cent). When grouped statistically, these data give a slightly skewed probability curve with a mode at +11,200 calories. The heat of solution of this quantity of oxygen was calculated to be +3,100 calories, leaving +6,950 calories as the average heat of reaction at 22°C.

A smaller number of measurements were made at 38°C. The average result for these eight determinations was +8,050 calories per gram molecule of oxygen, ±1,700 calories (21 per cent). The heat of solution at this temperature was +2,700 calories, leaving +5,350 calories as the average heat of reaction at 38°C.

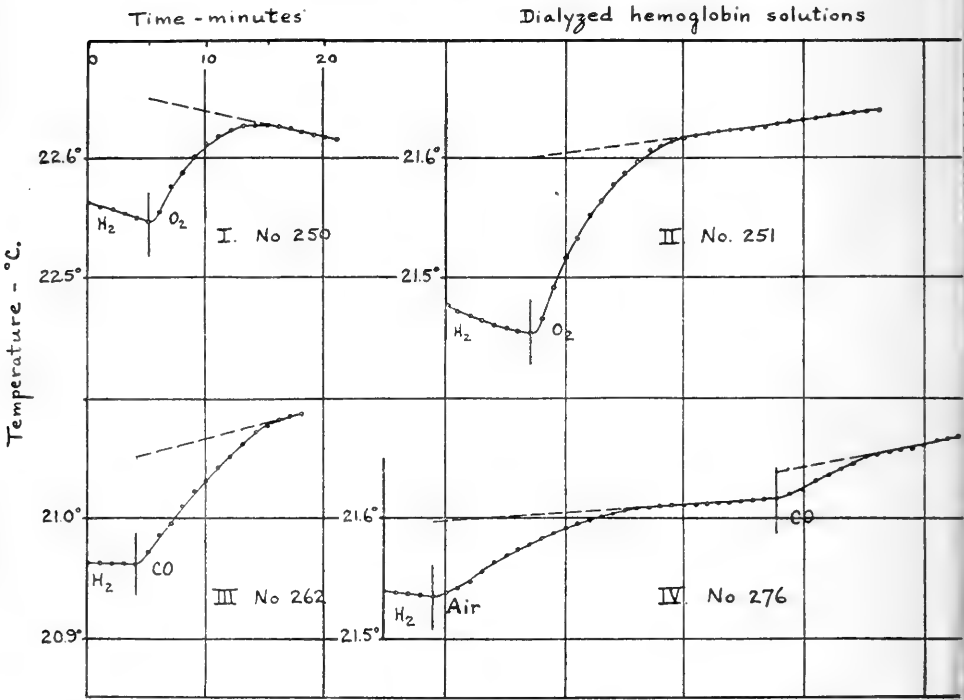

FIG. 4. Temperature changes recorded during thermochemical experiments with hemoglobin solutions.

For both temperatures together, the average was +6,650 calories.

The lower value at body temperature as compared to room temperature, while within the limit of error, is in agreement with the principles of thermodynamics. A smaller value of Q at body temperature was also obtained by du Bois-Reymond (17).

It should be noted that the amount of heat produced when hemoglobin unites with oxygen is not definitely correlated with

TABLE V.

Heats of Reaction of Oxygen with Hemoglobin.

(1) No.	(2) Solution.	(3) Temperature rise.	(4) Solution.	(5) Heat capacity.	(6) Initial HbO_2.	(7) Final HbO_2.	(8) Heat evolved per gram molecule HbO_2.
			22°C.				
		°C.	*cc.*	*calories/degree*	*per cent*	*per cent*	*calories*
236	Dialyzed whole blood.....	0.073	100	106		13.3	10,700
237	" corpuscles......	0.050	100	105		17.5	5,400
238	" "	0.056	100	106		13.0	8,900
238a	" "	0.070	97	102		15.3	8,700
239	" whole blood.....	0.065	95	101	1.2	13.5	9,400
240	" " "	0.059	94	99	3.7	13.4	10,700
244	Corpuscles + 0.9 per cent NaCl.	0.060	97	96	11.2	29.9	5,300
245	Dialyzed corpuscles......	0.099	97	99	6.4	22.1	10,400
247	" "	0.118	89	92	0.9	18.5	11,600
248	Corpuscles + 0.9 per cent NaCl.	0.254	97	93	0.6	37.3	11,100
249	Dialyzed corpuscles......	0.078	97	105	0.8	11.3	13,400
250	" "	0.103	97	103	1.4	17.1	11,600
251	" "	0.145	97	99	1.1	23.3	11,100
252	" "	0.100	97	103	1.6	17.0	11,500
253	" "	0.143	97	102	1.3	19.2	14,000
254	Corpuscles + 0.9 per cent NaCl.	0.148	100	100	3.1	23.0	12,400
255	Whole blood.............	0.080	95	99	2.9	19.1	8,600
256	Dialyzed corpuscles.......	0.105	97	103	0.9	17.1	11,500
259	" " + 0.002 M KH_2PO_4........	0.058	97	103	1.0	16.3	6,700
262	Dialyzed corpuscles + 0.003M KH_2PO_4.........	0.076	97	104	1.1	12.1	12,800
263	Dialyzed corpuscles......	0.114	104	110	1.0	16.4	13,000
264	" " + 0.074 N $NaHCO_3$........	0.074	70	74	2.3	14.5	11,600
265	Dialyzed corpuscles + 0.068 N $NaHCO_3$........	0.045	89	96	1.7	12.0	7,900
276	Dialyzed corpuscles......	0.063	96	101	8.5	18.4	11,200
278	" "	0.070	97	103	1.4	15.7	8,700
281	" "	0.086	97	103	1.0	16.0	10,000
281a	" " + 0.060 N KOH	0.075	97	102	2.5	15.4	10,200
283	Dialyzed corpuscles......	0.079	94	99	2.0	15.1	10,600
284	" "	0.100	88	89	5.7	22.3	10,200

TABLE V—*Concluded.*

(1) No.	(2) Solution.	(3) Temperature rise.	(4) Solution.	(5) Heat capacity.	(6) Initial HbO_2.	(7) Final HbO_2.	(8) Heat evolved per gram molecule HbO_2.
	22°C.—Concluded.						
		°C.	*cc.*	*calories/degree*	*per cent*	*per cent*	*calories*
286	Dialyzed corpuscles + 0.046 N lactic acid......	0.109	102	104	1.6	20.7	10,100
287	Dialyzed corpuscles......	0.132	88	90	1.4	21.6	11,200
288	" " + 0.046 N lactic acid......	0.076	99	106	4.2	14.6	13,100
289	Whole blood..............	0.048	95	103	5.0	13.7	9,900
289 a	" "	0.026	97	104	4.6	14.8	4,600
290	" "	0.080	94	100	5.3	15.6	13,800
	38°C.						
293	Dialyzed corpuscles......	0.094	98	102	0.3	20.8	7,800
293 a	" "	0.049	95	99	13.5	20.8	11,700
294	" "	0.064	67	71	1.9	19.3	6,500
294 a	" "	0.074	85	89	5.5	19.0	9,600
296	" "	0.081	97	103	1.0	18.0	8,400
296 a	" "	0.066	97	103	1.1	18.8	6,600
296 b	" "	0.086	92	94	1.2	23.0	6,700
297	Corpuscles + 0.9 per cent NaCl..................	0.151	96	93	1.9	36.4	7,100

Formula for calculation: $$(8) = \frac{(3) \times (5) \times 16{,}700 \times 100}{(4) \times [(7)-(6)]}$$

the presence of salts, acids, alkalies, or other proteins. Moreover, the age, dilution and previous treatment of the solutions, and substitutions of air for pure oxygen have no controlling influence. The latter portion of the oxygenation evolves as much energy as the total oxidation; the strict proportionality between combined oxygen and temperature production is shown in Fig. 5.

The heat of solution of the oxygen merely dissolved in blood is negligible, since the solubility of oxygen is so small that no heat of solution in water could be demonstrated directly. Moreover, it is nearly compensated by the displacement of hydrogen from solution.

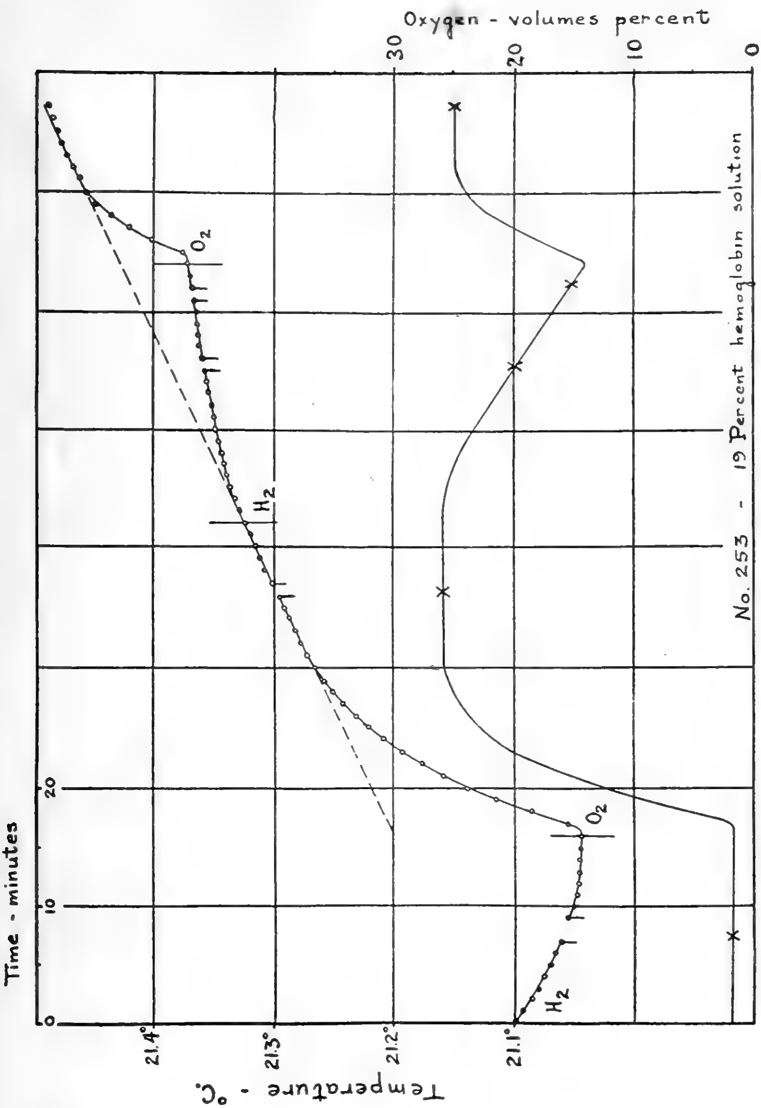

Fig. 5. Changes in temperature and in oxygen saturation during an experiment with hemoglobin

TABLE VI.

Heat of Reaction of CO with Hemoglobin.

(1) No.	(2) Solution.	(3) CO mixture used.	(4) Temperature rise.	(5) Solution.	(6) Heat capacity.	(7) Initial HbO_2.	(8) Final HbCO.	(9) Heat developed per gram molecule HbCO.	(10) Average heat of reaction.
Evacuated.									
			°C.	cc.	$\frac{calories}{degree}$	*per cent*	*per cent*	*calories*	*calories*
266	Corpuscles + 0.9 per cent NaCl.	Illuminating gas.	0.108	87	90	1.6	16.2	12,800	
267	Dialyzed corpuscles.	" "	0.127	85	89	0.9	16.0	14,700	
269	Whole blood.	" "	0.165	94	97	3.8	16.9	21,700	
272	Dialyzed corpuscles.	CO from oxalic + sulfuric acid.	0.054	92	100	1.5	5.0	28,000	
274	Whole blood.	CO from formic + sulfuric acid.	0.060	69	72	4.6	14.0	11,100	14,700
275	Corpuscles + 0.9 per cent NaCl.	CO from formic + sulfuric acid.	0.152	94	97	2.5	16.5	18,700	
277	" + 0.9 " " "	CO from formic + sulfuric acid.	0.140	97	100	6.8	17.9	21,700	
282	Dialyzed corpuscles.	CO from formic + sulfuric acid.	0.088	97	102	1.5	15.1	11,350	

Oxygenated.									
276	Dialyzed corpuscles.	CO from formic + sulfuric acid.	0.022	94	98	18.4	18.4	2,080	3,000
283	" "	CO from formic + sulfuric acid.	0.030	91	96	15.1	15.1	3,500	
288	" "	CO from formic + sulfuric acid.	0.024	91	95	14.6	14.6	2,860	
297	Corpuscles + 0.9 per cent NaCl.	CO from formic + sulfuric acid.	0.083	90	92	36.4	36.4	3,700	

Formula for calculation: $(9) = \dfrac{(4) \times (6) \times 16{,}700 \times 100}{(5) \times [(8) - (7)]}$

The standard deviation of 22 per cent in these measurements is perhaps as significant as the magnitudes themselves. The preliminary experiments with inorganic reactions have indicated that an accuracy of about 1 per cent can be attained. That such an agreement was not obtained with whole blood, and that previous workers on this problem have not agreed, indicates that important influences still uncontrolled are at work.

The result obtained for the heat of reaction of oxygen with hemoglobin, approximately +7,000 calories, is lower than that obtained by Berthelot (14), Torup (15), Barcroft and Hill (16), or du Bois-Reymond (17).

Reaction of Carbon Monoxide with Hemoglobin Solutions.—Only a few measurements of the heat developed in this reaction were made, using gas generated from formic acid and sulfuric acid. Similar results were obtained with illuminating gas, which is composed chiefly of hydrogen, carbon monoxide, and hydrocarbons (Table VI). In all cases hydrogen was used to give a fore period (Fig. 4, Curve III). Control measurements with water showed no appreciable heat of solution.

The average value for eight measurements was +17,500 calories per gram molecule, ±5,600 calories (32 per cent). The heat of solution of a gram molecule of carbon monoxide is +2,800 calories, leaving +14,700 calories. This is very close to the result of Berthelot (14).

A series of four experiments in which carbon monoxide replaced oxygen gave a smaller development of heat, averaging +3,000 calories. The heats of solution of oxygen and carbon monoxide in this case compensate each other. If the comparative values for oxygen and carbon monoxide, +7,000 and +14,700 calories are correct, it is to be expected that +7,700 calories will develop when carbon monoxide replaces oxygen. That only +3,000 calories were evolved, indicates the importance of other factors which are either experimental or chemical.

Reaction of Carbon Dioxide with Defibrinated Blood.—The amount of energy liberated when carbon dioxide combined with solutions of dialyzed hemoglobin varied tremendously, depending upon the ionic equilibria of the solution. But when carbon dioxide combined with whole blood the results were remarkably uniform, even with the blood of different individuals. The course

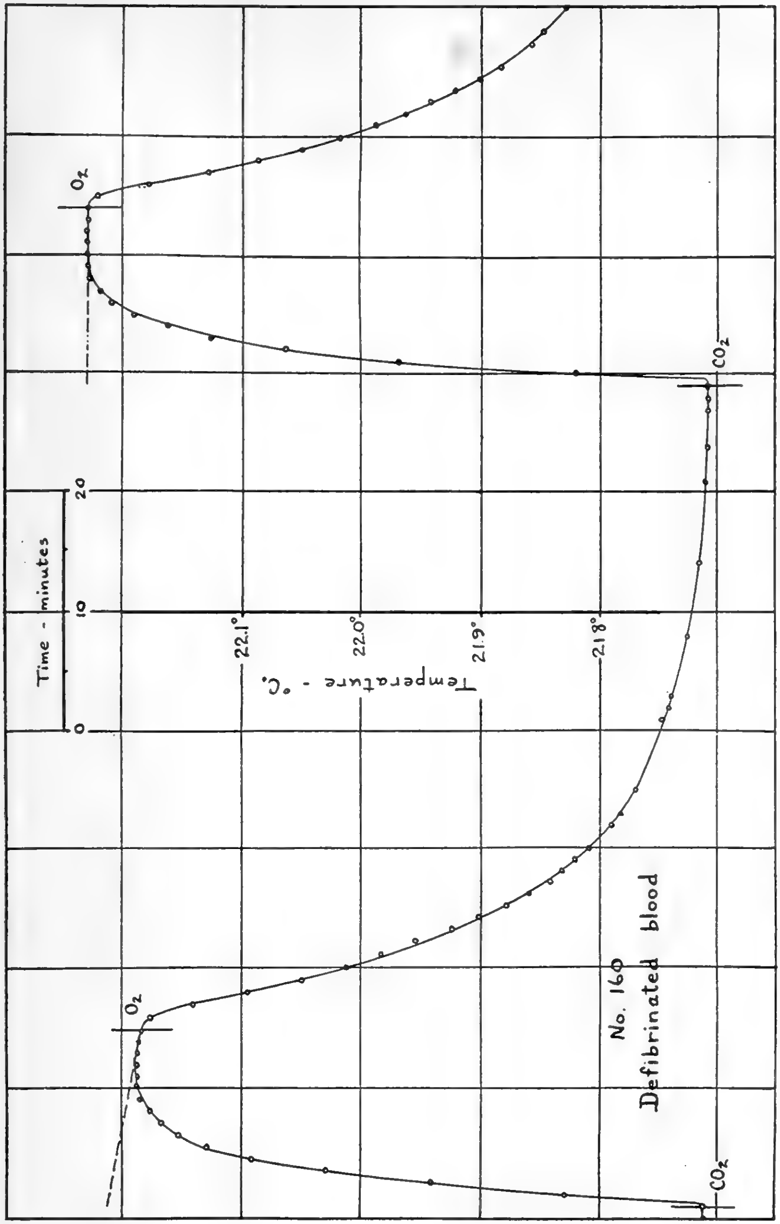

FIG. 6. Temperature changes during a thermochemical measurement using carbon dioxide and beef blood.

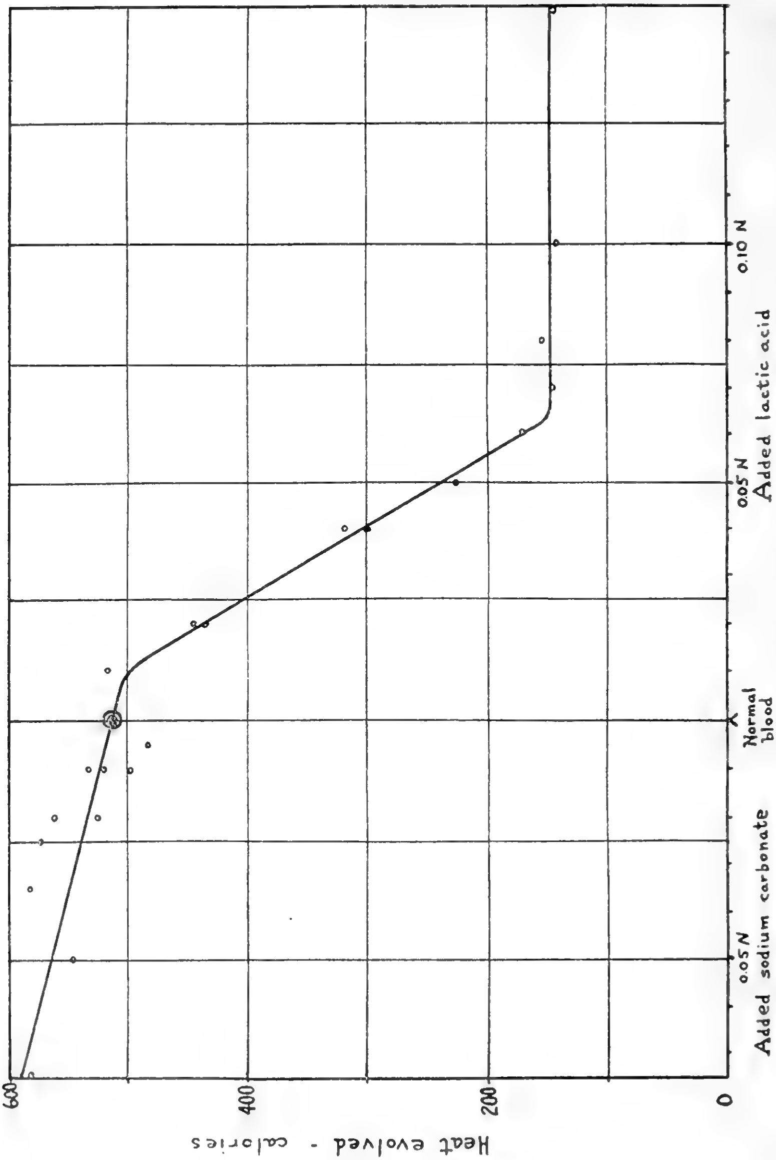

Fig. 7. Heat evolved by the saturation of 1 liter of beef blood with carbon dioxide at 22°C. The point for normal blood is an average of twenty-eight measurements.

of a typical experiment is given in Fig. 6. The blood was usually laked, octyl alcohol being present.

A series of twenty-eight measurements in which carbon dioxide was passed into fully oxygenated blood gave an average result of +513 calories per liter (standard deviation ±12 per cent). The defibrinated beef blood used included samples from eight different animals. The reverse reaction in ten measurements averaged −476 calories. When the blood was evacuated and saturated with hydrogen, the passage of carbon dioxide produced +486 calories per liter in six determinations.

Interesting results were obtained by adding known amounts of lactic acid or sodium carbonate to the blood; then passing carbon dioxide into it. The data are plotted in Fig. 7. It will be seen that 0.065 N lactic acid was sufficient to exhaust the acid-combining power of the blood. Added carbonate increased the heat production in strict proportion to the available alkali.

DISCUSSION.

The first worker to measure the heats of reaction of hemoglobin compounds, Berthelot (14), was interested in locating the heat production of the animal body. When he found that oxygen liberated considerable energy in combining with hemoglobin, he supposed that much heat was produced in the lungs, and calculated this to be about one-seventh of the human body's energy output. He suggested, however, that most of this portion of energy was used in the lungs in vaporizing water. Berthelot supposed that the liberation of carbon dioxide in the lungs involved little or no energy exchange.

From the results of the present experiments the heat exchange in respiration can be calculated. In saturating 1 liter of blood, carbon dioxide liberates 513 calories; oxygen liberates 84 calories. Complete saturation with oxygen requires an amount of gas which is only one-seventh of the carbon dioxide required for saturation, so that when the respiratory quotient is 1 these two reactions compensate almost exactly in chemical energy change. In the living body this is approximately true both in lungs and in tissues.

The dissociation equilibrium of oxyhemoglobin was first represented mathematically by Hüfner (24). Various discrepancies

between theory and fact have presented themselves, which have led to modifications of the Hüfner equation: $K = \frac{[Hb]\,[O_2]}{[HbO_2]}$. Nevertheless, the data of Barcroft and Roberts (25) showed that solutions of hemoglobin can be obtained for which K is constant at a given temperature for all oxygen tensions.

The data of Bert (26) and of Hüfner (27) show that the dissociation of oxyhemoglobin in whole blood increases with temperature. Barcroft and King (28) have plotted the actual curves at several temperatures, not only for blood but also for dialyzed hemoglobin solutions, and it is readily observed in their data that K, as defined above, is many times greater at 38°C. than at 14°C. The measurements of Barcroft and Hill (16) show the same change in dissociation with temperature.

Henri (29) was the first to suggest that by means of the isochore a relationship might be found for oxyhemoglobin between the temperature coefficient of K and the heat of reaction, Q. He showed that the heat of reaction ought to be very large, since K increases sixfold for a temperature change of 20°. Barcroft and Hill (16) again calculated this relationship, and found that Q must have a value of approximately 28,000 calories. These authors considered both K and Q as factors in a heterogeneous equilibrium; *viz.*, between gaseous oxygen and dissolved hemoglobin. In this paper their data are recalculated in terms of dissolved oxygen, corresponding to the form of our own data.

Calculations based upon the present series of experiments yield an average value for Q of roughly 7,000 calories. This series includes 43 determinations, as contrasted with 24 determinations by four other authors. Individual determinations in this series vary from 1,500 to 10,900 calories, while the results of others vary from 10,200 to 29,870 calories.

In Fig. 8 the variation of K with temperature is plotted, assuming that Q is 7,000 calories. In the same graph is plotted the experimental variation of K as found by Barcroft and Hill (16). In the first case K increases 1.4 times for 10°, in the second case 3.1 times. K is plotted here in arbitrary units, which represent approximately its true value when oxygen concentration is calculated in gram molecules.

It is evident that the experimental values of K and Q are not related by the simple isochore of van't Hoff. Careful criticism has revealed errors in the technique neither of our measurements of Q nor of Barcroft's (16, 28) measurements of the temperature coefficient of K which are sufficiently great to explain away the discrepancy. It is, therefore, a real one.

It seems worth while to recognize a number of unmeasured factors in hemoglobin systems. The measured value of Q on the

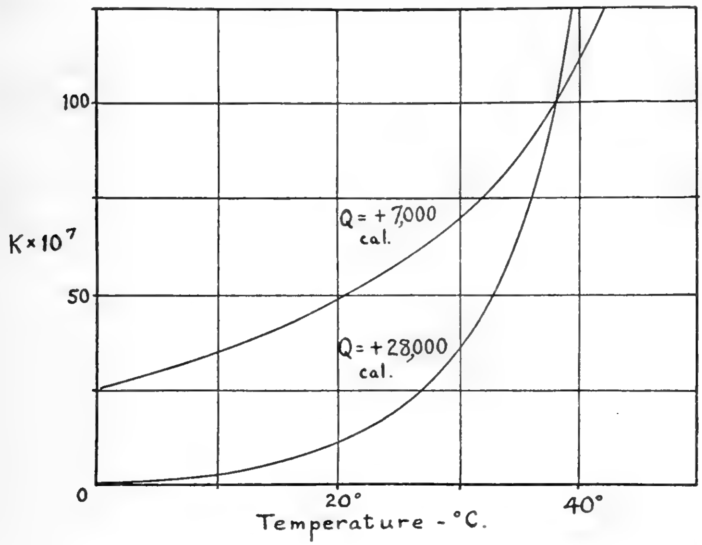

Fig. 8. Variation with temperature of the equilibrium constant for the dissociation of oxyhemoglobin, as calculated by van't Hoff's isochore.

one hand, undoubtedly includes energy derived from the aggregation and ionic dissociation of hemoglobin, at least one and perhaps both of which occur during oxygenation. The measured value of the temperature variation of K, on the other hand, probably includes the following factors:

1. Change in the solubility of oxygen with temperature. This is the only one of the factors which can be corrected for, and when calculations are applied to Barcroft's (16, 28) data, they are altered relatively slightly.

2. Change in the aggregation equilibria of hemoglobin and of oxyhemoglobin. While Hill (30) and Haldane (31) have been able to calculate the amount of aggregation of molecules which may take place when hemoglobin gives up oxygen, it is not known how this ratio of equilibrium constants varies with temperature.

3. Change in ionic dissociation of hemoglobin and of oxyhemoglobin. The deductions of Henderson (32) and the experiments of Adolph and Ferry (20) show that probably the ionization of hemoglobin changes its equilibrium with variation in combined oxygen. It is impossible to distinguish this change from the changes due to other shifts in ionic equilibria, all of which are probably influenced greatly by temperature.

4. The redistribution of cations associated with hemoglobin and with oxyhemoglobin. There is little doubt that each salt of either substance differs in its behavior toward oxygen.

5. The redistribution of all ions and molecules not chemically related to hemoglobin or oxyhemoglobin. This is at a minimum in dialyzed preparations. Barcroft (33) has drawn the conclusion that there are at least two ways in which the marked influence of salts is exerted; first, by changing the aggregation of hemoglobin, and secondly, by changing the ionization of hemoglobin. There may in addition be a direct influence of salts.

In the light of these considerations it is perhaps not surprising that simple theory and complex fact are apparently at variance. The discrepancies may be summarized for the present in the statement that the active mass of hemoglobin differs from its measurable properties in its behavior toward oxygen.

It is of interest to apply the isochore to the equilibrium between carbon monoxide and hemoglobin. The equation $k = \frac{[Hb]\ [CO]}{[HbCO]}$ has been shown by Haldane (31) and Hartridge (34) to be subject to the same conditions as that for oxyhemoglobin. By measuring the equilibrium when both oxygen and carbon monoxide are present in solution at several different temperatures, they showed that the change with temperature in the k for carbon monoxide is from 5 to 10 per cent higher than in the K for oxygen.

Applying the isochore to their data one would expect to find q for carbon monoxide hemoglobin approximately 8 per cent greater

than Q for oxyhemoglobin, or about 7,500 calories, if our average for Q is correct. Our measurements of q, in common with those of Berthelot (14) and of du Bois-Reymond (17), show that the actual q is very much larger than the heat of reaction for oxygen, about 15,000 calories. Reversing the use of the isochore, it is found that k increases rapidly with temperature in such a case. Moreover, the experiments in which carbon monoxide replaced oxygen which was already combined with hemoglobin show an evolution of heat, not of 500 calories nor of 8,000 calories, but of 3,000 calories.

The influences of acids, alkalies, salts, and dilution upon the dissociation of carbon monoxide hemoglobin are the same as those upon oxyhemoglobin (31, 34). The temperature effect and the energy of reaction are the only chemical properties studied so far that are known to differ for the two compounds; and these two quantities are not correlated in the expected manner in the case of either compound.

SUMMARY.

1. The technique and apparatus used in measuring the energy exchange in gas-liquid reactions are described.

2. The thermochemical method is shown to be useful in studying (1) the location of animal heat production, (2) the velocity of reactions, (3) the amount of oxygenation and reduction of hemoglobin, (4) the neutralizing power of solutions, and (5) the heat of reaction as applied in the use of van't Hoff's isochore and in the measurement of chemical affinity.

3. It is shown that neither the isochore nor the mass law can be applied directly to the oxyhemoglobin system, under the limitation of present analytical methods. Several unmeasured factors that occur in every hemoglobin system are suggested as partially responsible for this.

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THE PHYSIOLOGY OF THE PHENOLS.

I. A QUANTITATIVE METHOD FOR THE DETERMINATION OF PHENOLS IN THE BLOOD.

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Liver function and related problems have interested several workers in this laboratory during the past few years. As a part of this research program it seemed advisable to examine critically the conjugation of phenolic substances in the body. The mechanism of absorption, conjugation, distribution, and excretion can be studied when a suitable method is at hand. The method described and controlled below is sufficiently accurate to make possible a careful study of these phenolic substances in the blood and body tissues.

Benedict and Theis (1) in 1918 made an attempt to apply the urinary phenol method of Folin and Denis (3) to the blood. The principal modification consisted in a separate determination of uric acid by Benedict's method and an addition of sodium bisulfite to stabilize the color. Their conclusion from an examination of a number of pathological cases was that blood contains an average of 4.70 mg. of phenol per 100 cc., that it contains no conjugated phenols, and that the polyphenols appear to represent about one-third of the total phenols.

From all the experimental evidence presented by other workers we must regard as an established fact that the phenols are formed in the intestinal tract by bacterial decomposition of tyrosine, and that they are excreted largely in the urine. Only a small percentage is excreted with the feces (4). Also, it has been conclusively shown that a large percentage of the urinary phenols exists in the conjugated form (9). Since we have no reason to believe that conjugation occurs either in the kidneys or in the bladder we may

safely assume that at some time or other the conjugated phenols pass through the blood prior to excretion. It should, therefore, be possible to demonstrate and measure these substances in the blood stream.

An examination of Benedict and Theis' method shows that there are several factors which may well prevent the demonstration of minute quantities of conjugated phenols. The separate determination of uric acid not only complicates the method considerably, but involves an unavoidable experimental error. The boiling of the blood filtrate down to less than half the volume is the process primarily responsible for their inability to demonstrate conjugated phenols in the blood. Benedict and Theis state that only 85 per cent of resorcinol added to the blood is recovered by their method and that phenol itself added to the blood disappears completely during the boiling of the filtrates. There are sufficient reasons to believe (9) that the bulk of the conjugated phenols is made up of two very volatile phenols—*p*-cresol and phenol—and it is, therefore, logical to assume that the boiling of the filtrates in Benedict and Theis' method is responsible for the disappearance of the conjugated phenols.

However, it is possible to modify Folin and Denis' original method so that it may be applied to blood. It seemed important to eliminate heat as a factor in the precipitation of the blood proteins and in the concentration of the filtrate. The precipitation of proteins with tungstic acid, as described by Folin and Wu (5) is an excellent substitute for the boiling acetic acid; the precipitation is fully as complete, and the reaction takes place at room temperature. In order to remove the last traces of protein, aluminum cream is added. It was further found that with a standard of 5 mg. of phenol per 100 cc. set at 20 in the Duboscq colorimeter, the color developed in the blood filtrate is easily and accurately readable. This eliminates the concentration of the filtrate by boiling as carried out by Benedict and Theis. Another objection to Benedict's method is the separate determination of uric acid by an entirely different and time-consuming method. In order to simplify this step a number of uric acid precipitants, such as Morris' (8) zinc salt and Curtman and Lehrman's (2) nickel salt were tried, but, while the precipitation of uric acid is complete, the excess zinc or nickel is difficult to remove from the solution, and

if not removed interferes with the final color reaction. Folin uses silver lactate in lactic acid as a precipitant for uric acid, but the difficulty with lactic acid lies in the fact that it gives a blue color with Folin's phenol reagent; a fact which was overlooked by Folin and may in part be responsible for his high figures. Nevertheless, this method of precipitating the uric acid was retained, but the error caused thereby is now corrected by addition of a corresponding amount of lactic acid to the standard. Benedict's innovation of stabilizing the color by addition of sodium sulfite

TABLE I.

*Recovery of Phenols Added to Blood.**

Before addition of phenols.	After addition of 5.81 mg. *p*-cresol† to 100 cc. of blood.
mg. per 1,000 cc.	*mg. per 1,000 cc.*
24.6	74.0
29.1	78.9
25.6	76.0
	Addition of 5 mg. phenol.
25.2	75.0

* The blood proteins must be precipitated immediately after the addition, as prolonged standing destroys a part of the phenols.

† 5.81 mg. of *p*-cresol are equal in color production to 5 mg. of phenol. The colorimeter reading is expressed as phenol.

TABLE II.

Accuracy of Separate Simultaneous Determinations of Phenol.

Determination.	Dog 1.	Dog 2.	Dog 3.
First............	28.4	36.25	31.0
Second..........	28.75	37.1	30.6
Third...........	28.0	37.50	

is omitted. Due to the more complete precipitation of the proteins and the higher dilution in which the readings are made, the dirty green tinge which sometimes occurs with Benedict's method does not appear. Finally another change has been made in the method: due to the higher dilution no addition of water to make up to 50 or 100 cc. as in Folin's or Benedict's methods is necessary, and there is substituted a manipulation of the final filtrate in graduated test-tubes which greatly increases the accuracy of the method and makes possible the detection of conjugated phenols in very minute quantities.

Method.

The entire method is very simple and rapid. 10 cc. of blood are added to 50 cc. of distilled water in a 100 cc. Erlenmeyer flask. Then 10 cc. of 10 per cent sodium tungstate and 10 cc. of $\frac{2}{3}$ N sulfuric acid are added, the flask is closed with the thumb or rubber stopper, and vigorously shaken for a few seconds. To precipitate the proteins completely 10 cc. of aluminum cream are added and the flask is again shaken. The contents are transferred to a 100 cc. centrifuge tube and centrifugalized for 45 minutes. The supernatant fluid is filtered to the 45 cc. mark in a narrow 50 cc. graduate, 5 cc. of a 5 per cent solution of silver lactate in 5 per cent lactic acid are added, and the graduate is well shaken for 1 minute. After centrifugalization and filtration, the filtrate is ready to be examined for phenols. This last step is carried out as follows: only two narrow test-tubes are required —one graduated at 15 cc. and the other at 10 cc.—in which both the total and the free phenols are determined. Thus, any error due to the graduation of two sets of test-tubes is avoided. The procedure for the determination of *free* phenols is this: the 15 cc. tube is filled to the mark with the filtrate, 1 cc. of the phenol reagent[1] is added, and the tube is shaken. The excess silver precipitates out, the solution is filtered to the mark into the 10 cc. tube, and 5 cc. of 20 per cent sodium carbonate are added. This solution is now transferred to another test-tube in which the color develops to its maximum in about 20 minutes. The two graduated test-tubes are meanwhile used for the determination of *total* phenols. The 15 cc. tube is again filled to the mark with the same filtrate, 5 drops of concentrated HCl are added, and the tube is placed in a water bath at 100°C. for 10 minutes. Boiling of the contents of the tube is avoided and no loss of volatile phenols occurs, as was shown by repeating the determination with known amounts of phenol. If the tube has a diameter of 14 to 15 mm.,

[1] Bell's modification of the reagent (Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 508) is used, since the HCl which it contains is needed for the precipitation of the excess silver. It contains: 100 gm. of sodium tungstate, 20 gm. of phosphomolybdic acid, 50 cc. of phosphoric acid 85 per cent, 100 cc. of concentrated HCl. This is gently refluxed for 2 hours with 750 cc. of water, and at the end of the period of heating made up to 1,000 cc.

TABLE III.

Removal of Uric Acid by Precipitation.

Before addition of uric acid.	After addition of 5 mg. of uric acid to 100 cc. of blood.
Total phenols per 1,000 cc.	Total phenols per 1,000 cc.
mg.	*mg.*
23.5	23.8
25.5	25.5
29.0	29.5

TABLE IV.

Influence of Amino-Acids Normally Present in the Blood.

Before addition of casein digest.	After addition of casein digest (20 mg. of amino N to 100 cc.)
Total phenols per 1,000 cc.	Total phenols per 1,000 cc.
mg.	*mg.*
34.2	36.8
31.4	35.0
24.6	27.5

TABLE V.

Demonstration of Conjugated Phenols in Human and Dog's Blood by the Author's Method.*

Blood.	Total.	Free.	Conjugated.	
	mg. per 1,000 cc.	*mg. per 1,000 cc.*	*mg. per 1,000 cc.*	*per cent*
Human.				
Decompensated heart.....	31.2	28.5	2.7	8.6
Fracture of arm..........	37.2	33.2	4.0	10.7
Normal..................	42.8	38.0	4.8	11.2
Dog.				
Normal..................	29.4	28.2	1.2	4.1
	32.5	30.1	2.4	7.4
	38.1	35.2	2.9	7.6
	34.0	32.5	1.5	4.4
	27.4	24.4	3.0	10.9

* This table is not intended to give average values for human blood (for which purpose a much greater number of determinations must be made) but merely to demonstrate the presence of conjugated phenols.

the volume of the contents on cooling at the end of exactly 10 minutes is back to the graduation mark, so that no adjustment of volume is necessary. Then 1 cc. of the phenol reagent is added and the solution is treated in the same way as in the determination of free phenols. The difference between the total phenols and the free phenols represents conjugated phenols.

The standard is prepared as follows: 5 cc. of the stock solution of resorcinol (Benedict and Theis, 1), containing 5.81 mg. are placed in a 100 cc. volumetric flask, 0.5 cc. of concentrated HCl and 10 cc. of the silver lactate-lactic acid solution added, centrifugalized or filtered, and the filtrate is manipulated in the graduated test-tubes in the same manner as the blood filtrate in the determination of free phenols.

Recently there have been published several criticisms of Folin's phenol reagent (6, 7, 9), from which it appears that this reagent gives a blue color with a great many substances, and that it is by no means specific for phenols and the closely allied hydroxy-acids. It must be said, however, that most of these substances are normally not present in the blood, and that the normal constituents of the blood which interfere with the reaction can either be readily removed (uric acid, Table III), or are in such small concentration (amino-acids, Table IV), that their presence accounts, at best, for only a negligible fraction of the color developed. Further, in experiments such as reported in the later papers of this series, where we are dealing with the measurement of injected or ingested phenols, the color-producing effect of these interfering substances is entirely eliminated by a preliminary determination.

SUMMARY.

1. A method is described for the determination of phenolic substances in blood, which is based on Folin's method for the determination of phenolic substances in urine.

2. Contrary to the opinion of Benedict and Theis (1) conjugated phenols are present in human as well as in dogs' blood and can be demonstrated with the above method.

I am indebted to Professor W. R. Bloor and Dr. C. L. A. Schmidt of the Department of Biochemistry for their helpful advice and criticism in the development of this method.

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THE PHYSIOLOGY OF THE PHENOLS.

II. ABSORPTION, CONJUGATION, AND EXCRETION.

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With a suitable method at hand for the quantitative estimation of phenolic substances in the blood we took up a study and review of the factors concerned in the normal and abnormal metabolism of phenols in the body, directing particular attention to the liver. Many investigators have studied this question and have marshalled experiments to prove or disprove that the conjugation of phenols takes place in the liver, or again in the kidney, or elsewhere in the body. The best evidence to date is in favor of conjugation taking place in the liver, but in the face of contradictory experiments it must be admitted that much of this evidence is indirect and the question is at least open to debate. We may now review some of the recent work which concerns our thesis.

Baumann (1) noted a temporary accumulation of conjugated phenols in the liver of a dog poisoned with phenol, and showed that he could obtain nineteen times more phenol-sulfuric acid from that organ than from other organs or the blood. He suggested that the liver is the organ primarily concerned with the synthesis. Christiani and Baumann (4) tied off the ureters of a dog and showed that no accumulation of ethereal sulfates occurs in the blood. From this they concluded that, at least when the ureters are tied, the kidneys take no part in the conjugation of phenols. In other and more convincing experiments they ligatured all renal vessels and poisoned the dog with phenol. Synthesis occurred to the same extent as with normal dogs. From these experiments they concluded that if the kidneys are at all concerned with this reaction they are concerned with it to a negligible extent only. In the pursuit of the question of the participation of the kidneys in this synthesis Baumann and Herter (2) had already tried to perfuse the kidneys with blood containing phenol and sodium sulfate and had been unable to show any synthesis. Kochs (14) ground up liver, kidney, pancreas, and muscle, and added phenol plus sodium

sulfate. He states that a moderate amount of conjugation was demonstrable in each case, and that negative results were obtained with thymus gland. Landi (15) repeated Kochs' experiments but could find no conjugation. From perfusion experiments with some of the organs he concludes that the intestine is the seat of the synthetic process. Lang (16) found small amounts of conjugated phenols in the urine of geese whose livers he had extirpated, and thinks that the liver is not the exclusive, although the most important, organ of conjugation. Herter and Wakeman (11) added equal amounts of phenol to blood and ground brain, muscle, kidney, and liver, and showed an increasing disappearance of phenols in the order given. They do not believe, however, that this disappearance is due to a conjugation with sulfuric acid; rather they favor a chemical destruction or a "loose combination of the phenol molecules with the molecules of the substance." Salta (19) thought that he could determine the place of conjugation by an analysis of various organs for phenol-sulfuric acid. The largest amount was found in the liver. Then follow in decreasing amounts in the order given: muscles, lungs, intestine, stomach, nerves. No conjugated phenols were found in the brain. From this he reasons that all these organs play a part in the synthesis. Embden and Glaessner (6), in a number of carefully controlled perfusion experiments, show that the conjugation takes place almost exclusively in the liver, although very small amounts of ethereal sulfates were found in the lungs and kidneys.

The work of Herter and Wakeman (11) is sometimes cited (5) as proof that other organs than the liver, *i.e.*, the intestinal epithelium, kidney, muscle, brain, blood, etc., have the ability, although to a lesser extent, to conjugate phenols with sulfuric and glucuronic acids. This statement, which is not in accordance with observable facts, is based on a misapprehension of the work of the first named authors. Although their experiments showed a disappearance of phenols when known amounts were added to and left in contact for some time with ground liver, muscle, kidney, etc., there is no evidence that this disappearance is due to a synthesis of phenol-sulfuric or glucuronic acids. In fact, Herter and Wakeman themselves state[1] that "the synthesis of indoxyl potassium sulphate cannot be accomplished by extirpated cells. It also seems improbable that the dead cells convert phenol into phenol-sulphuric acid." They then attempt to show[2] that this destruction of phenols is not in the nature of an oxidative process since they were unable to recover such oxidation products of phenol

[1] Herter and Wakeman (11), p. 317.

[2] Herter and Wakeman (11), pp. 317–318.

as hydroquinone or pyrocatechol. By subjecting liver pulp to various ferment-destroying agents (alcohol, boiling water, hot air, bichloride of mercury) and finding no decrease in the behavior towards phenol, these authors consider it highly improbable that ferments play any important rôle in this disposal of phenols, and they finally speculate[3] as to the probability of some "loose combination of the phenol molecules with the molecules of the cell substance."

It should be strongly emphasized that there is little experimental evidence for the statement that other organs than the liver have this ability to conjugate phenols. If the transformation of phenols by dead tissues is due to conjugation, it should be possible to hydrolyze these conjugated phenols by the addition of

TABLE VI.

Blood and Liver Incubated with Phenols.

Blood blank.	Blood + 5 mg. phenol per 100 cc. and 2 hrs. incubation.	Same + 5 mg. $K_2S_2O_8$ per 100 cc.	Same hydrolyzed with 5 drops HCl and heat.
mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
2.8	7.6	7.4	7.45
3.2	7.9	8.2	8.1
Liver as above.			
6.6	9.8	10.0	9.4
7.25	10.5	10.1	10.6

a few drops of HCl and the application of heat, and so recover the total amount of added phenol. That this is not the case is shown in Table VI in which are recorded the results of an experiment similar to those of Herter and Wakeman. Instead of using Millon's reagent the method described in the first paper of this series was used.

Known amounts of ingested phenols cannot be completely recovered in the urine or feces, and this loss is probably due to a similar reaction by which organ pulp disposes of phenol, *i.e.*, chemical oxidation, ferment action, or Herter and Wakeman's "loose combination."

[3] Herter and Wakeman (11), pp. 319–320.

Tauber (22) fed phenol to a dog and found that as the dose was decreased, the amount of phenol so "oxidized" was increased. On feeding 240 mg. of phenol in water *per os*, he found that 53 per cent of the ingested phenol was oxidized in the body through oxalic acid to carbon dioxide. Dubin (5) found that 68.7 per cent of ingested phenol and 50.6 per cent of *p*-cresol could be recovered from the urine. When tyrosine was given, only 17.7 per cent of the amount of phenol corresponding to the amount of tyrosine administered was recovered. Friedländer (9) recovered only 25 per cent of ingested cresol from the urine of his dogs. Numerous other workers have noted the fact that ingested phenols cannot be quantitatively recovered from the excretions. Siegfried and Zimmermann (20) fed *p*-cresol to a dog and recovered both *p*-cresol and phenol, the sum of both amounting to 32 to 48 per cent of the *p*-cresol fed. Jonescu (13) administered cresols to dogs and noted that they were oxidized in the order of ascending toxicity. The percentage oxidation was as follows: *m*-cresol 50 to 53 per cent, *o*-cresol 65 to 69 per cent, and *p*-cresol 73 to 76.5 per cent. As an example of the powerful oxidation to which phenols may be subjected in the organism, Jaffé (12) has shown that the ingestion of benzene increases the urinary output of muconic acid, and suggests that this is due to the cleavage of the benzene ring into a straight chain compound. The whole subject of oxidases and phenolases is exhaustively treated in Oppenheimer's textbook (18) and need here not be further considered. ("Die Wirkung dieser Phenolasen ist neben der Oxidation, die niemals tiefgreifend ist, häufig eine Kondensation mehrerer Moleküle. Das hängt mit ihrem physiologischen Zwecke der Schaffung unlöslicher Stoffe zusammen.")

This oxidizing action on the phenols which has been noted by Tauber to increase as the amount of volatile phenol present decreased, would explain why normally there are such small amounts of conjugated phenols in the blood: the volatile phenols produced in the intestinal tract are probably to a large extent rapidly oxidized by the intestinal mucosa and the liver—and those which escape this process are *completely* conjugated by the liver. When the oxidizing mechanism is overwhelmed by large amounts of phenol, as occurs when phenol is ingested, more of that substance reaches the hepatic tissues unchanged and the relative amount of conjugated phenols is greater. Ordinarily this oxidizing mechanism is sufficient and much larger amounts of volatile phenols than are normally produced in the intestine are oxidized within a few minutes. This serves to strengthen the argument that free volatile phenols as such cannot remain in the blood stream for any considerable period.

It now appears probable that in its effort to dispose of phenol which reaches the circulation from the intestinal tract, the body

makes use of two methods: one a process of oxidation which takes place to a considerable extent in the epithelial lining of the intestinal tract and in the liver and to a lesser extent in the other organs; the other a process of conjugation with sulfuric and glucuronic acids which takes place, as we shall later show, exclusively in the liver.

As regards the nature of the phenolic substances in the blood, there is no reason to suppose that they are essentially different from those in the urine. The most important literature dealing with that subject has been summarized by Folin and Denis (8), Dubin (5), and Tisdall (23).

EXPERIMENTAL OBSERVATIONS.

It seemed necessary to control diet factors in these experiments because of the observations of Underhill and Simpson (24) to the effect that urinary phenols vary directly with the protein intake, and with an increase in output of total phenols the percentage of free phenols remains constant. Some similar observations are recorded in Table VII which gives controls for the various diets under laboratory conditions.

Dogs weighing 20 to 35 pounds were used in these experiments. Mixed diet indicates a liberal amount of mixture of table scraps including cooked meat, macaroni, potatoes, bread, and bones.

The most important volatile phenols present in the body are *p*-cresol and phenol (7) and we, therefore, limited out experiments to these two substances focussing our attention upon *p*-cresol, as we found that complete conjugation occurs with it more rapidly than with any other phenolic substance investigated. At first we injected known amounts of phenol in a watery solution into the jugular vein of the animal and analyzed for total and free phenols the blood drawn just prior to and at stated intervals after the injection. Charts A and B give typical results of some of these early experiments. These experiments show that 5 minutes after the injection of 500 mg. of *p*-cresol or phenol the "theoretical concentration" of about 500 mg. per 1,000 cc. of blood has fallen to 80 mg. per 1,000 cc. We say "theoretical" advisedly, for the disappearance of phenols from the blood stream is so rapid that, due to the time lost during the slow injection this concentration is never approximated. The dog weighed 24 pounds and we may

assume an approximate blood volume of 1,000 cc. This almost instantaneous disappearance of injected phenols is, we believe, due to two factors; a rapid and relatively uniform distribution throughout all tissues of the body, and oxidative processes also occurring throughout the body although not with equal rapidity in all tissues. The amount of *conjugated phenols* in the blood 5 minutes after the injection is only slightly greater than before injection, but it increases rapidly to a maximum about 1 hour after injection. At the end of the hour the *free phenols* have reached their pre-

TABLE VII.

Diet Factors and Blood Phenols.

Dog.	Total phenols per 1,000 cc. of blood.	Conjugated phenols.	Total phenols per 1,000 cc. of blood.	Conjugated phenols.
	Exclusive carbohydrate diet.		High protein diet.	
	mg.	*per cent*	*mg.*	*per cent*
21-56	26.5	7.0	39.4	8.0
21-95	23.4	5.5	42.2	7.0
21-95	24.0	6.4	45.9	8.4
21-98	31.7	7.4	38.8	6.6
21-80	25.0	8.0	43.0	7.8
	5 to 8 days fasting.		Mixed diet.	
21-98	28.1	9.0	36.8	4.0
21-105	23.5	6.3	37.9	6.1
21-80	29.0	7.0	28.2	11.0
21-80	32.4	7.4	35.0	5.8

injection level and the conjugated phenols fall slowly (6 to 12 hours) back to their normal level.

As these curves are typical of a large number of determinations made on different dogs, it can be safely concluded from their examination that *p*-cresol is the more quickly oxidized or conjugated substance, for the fall of the free phenols back to the original level occurs sooner in every experiment.

The injection of such large amounts of highly poisonous substances invariably produces severe systemic reactions. Immediately after injection the pupils are widely dilated, the animal has convulsions and is unable to stand. In most cases the animal becomes clinically normal within 5 minutes. In some cases

animals which have previously reacted moderately to repeated injections will not survive the unit dose. These intravenous injections are always dangerous and may destroy a valuable standardized animal. Because of this difficulty we examined the

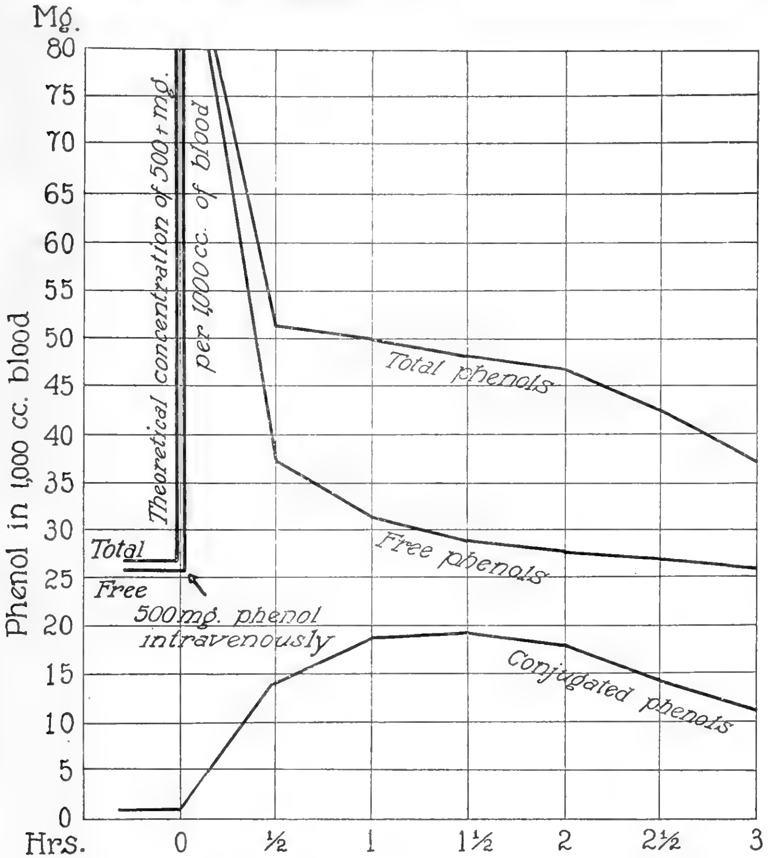

Chart A.

reaction to phenol solutions administered by stomach tube, and found that doses lethal for intravenous injection often produced no symptoms when given by stomach. An examination of Chart C shows that absorption from the intestine is sufficiently rapid and regular to be substituted in our experiments for intravenous in-

jection. In fact, the ingestion of phenols is superior to the injection as it will be seen that complete conjugation of ingested phenols is more conspicuous. This, no doubt, is due to the fact that all the ingested phenols must pass through the liver before

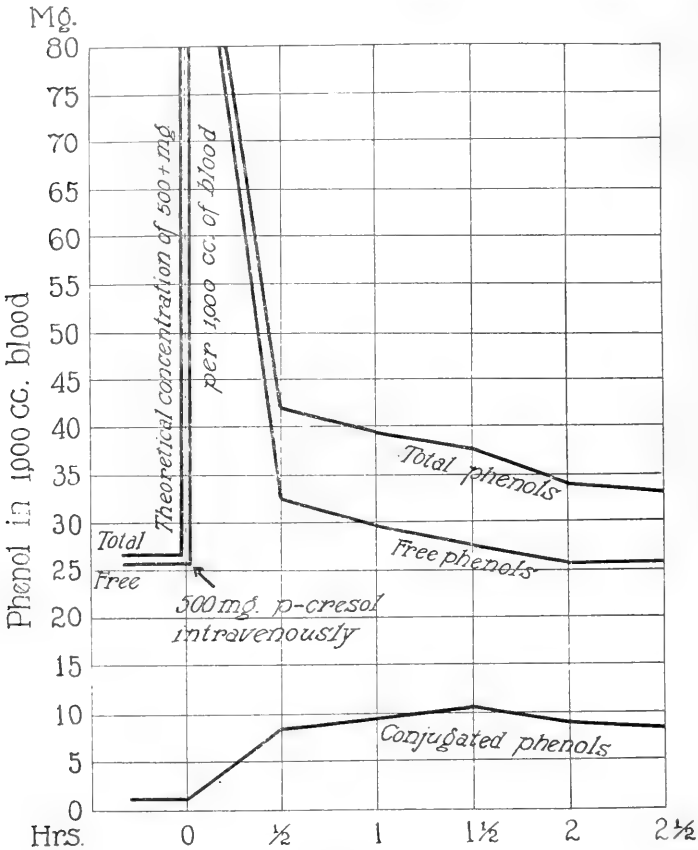

Chart B.

reaching the general circulation, while injected phenols are first distributed to all the tissues of the body and reach the liver much more slowly. The slight irregularities which sometimes occur in the ascending limb of the curve may be due to differences in stomach and intestinal absorption. In this connection it is interesting

to recall the statement of Sollmann and Hanzlik (21) that the absorption of phenols is not a steady process, because of a slowing of the local intestinal circulation by these substances. A standard dose of 50 mg. in 10 cc. of water per pound body weight given by stomach tube has been used in all experiments reported in this paper except where otherwise stated.

A closer examination of the curves obtained by plotting the results of consecutive blood analyses reveals the following: immediately after *ingestion* both the total and free phenols begin to rise—the free somewhat slower than the total. The total phenols reach their greatest concentration between $\frac{1}{2}$ and 1 hour after

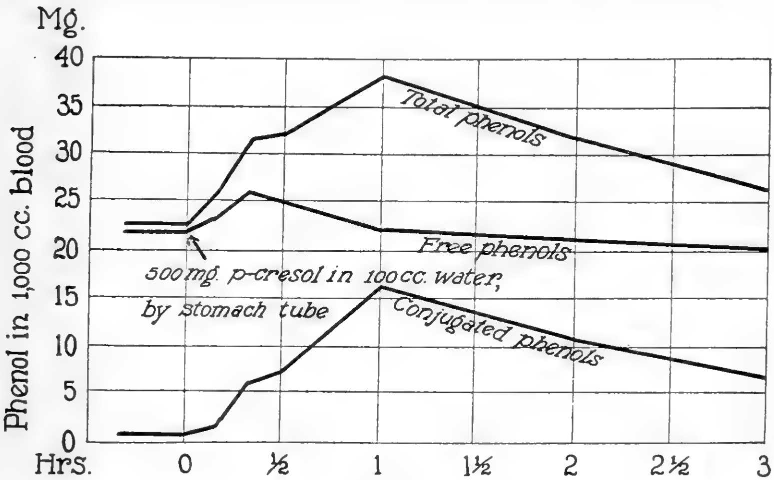

CHART C.

ingestion and after that time decline very slowly (6 to 12 hours) to their former level. The free phenols, after a slight rise within the first 20 or 30 minutes, quickly return to their old level ($\frac{1}{2}$ to 1 hour). In many cases the free phenols fall somewhat below that level. The resulting differences between the total and free phenols represent conjugated phenols which are plotted at the bottom of the charts. It will be seen that the conjugated phenols reach their greatest concentration 1 hour after ingestion and at this time the conjugation of ingested phenols is *complete;* an observation which corroborates Baumann (3), who showed that 1 hour after the intravenous injection of phenols the free phenols dis-

appeared from the system. After reaching the greatest concentration within 1 hour, the conjugated phenols are slowly excreted, corresponding to the slow fall of the total phenols.

From the fact that in a healthy animal the conjugation of very large amounts of ingested phenols is complete within 1 or 2 hours, it would appear unlikely that *free volatile phenols* are normally present in the blood. The conjugation of the small amount of volatile phenols produced in the intestine must be very rapid within the liver, and it is improbable that any free volatile phenols reach the general circulation under normal physiological conditions.

The bulk of the blue color (90 per cent and more) which is produced by Folin's reagent with the blood filtrate is due to substances other than true phenols. It may be due, among others, to hydroxy-acids and unidentified protein decomposition products as well as to carbohydrates and related substances (10, 17); the remainder of 10 per cent or less is produced by the conjugated phenols.

These three tables (Tables VIII, IX, and X) supplement each other and bring out several interesting points. The various organs compared with blood (Table VIII) contain substances which react with the phenol reagent and give larger figures for their "phenol" content. We have no reason to suppose that these reacting substances are true phenols.

The injection of phenol (Table IX) gives an immediate increase in phenol content of the blood and tissues. There is probably a pretty uniform distribution of the phenols in the blood and tissues but we record in analyses about 40 mg. increase in blood and 25 to 30 mg. increase in the parenchymatous organs.

After an hour's interval following a phenol injection (Table X) we note a uniform distribution of conjugated phenols in the blood and organs.

From these facts we wish to assume that free phenols, when injected into the blood stream, are distributed throughout the living tissues. After conjugation by the liver they again diffuse out to the tissues before being excreted (Table X). When phenols are given by stomach no such distribution of *free phenols* occurs because they are conjugated by the liver before reaching the general circulation. This last fact explains why doses of phenol,

lethal when injected into the blood stream, show no effects when given by stomach tube, but are lethal once again when administered by stomach tube to a dog whose liver has been seriously injured.

TABLE VIII.

Distemper Dog, Killed with Chloroform.

Organ.	Total phenols per 1,000 gm.	Free phenols per 1,000 gm.	Conjugated phenols.
	mg.	*mg.*	*per cent*
Blood	22.3	20.8	1.5
Kidneys	65.8	65.8	0.0
Liver	71.3	70.1	1.2
Spleen	59.4	57.9	1.5

TABLE IX.

Dog Killed by Injection of Phenol into Jugular Vein, Death 5 Minutes after Injection.

Organ.	Total phenols per 1,000 gm.	Free phenols per 1,000 gm.	Conjugated phenols.
	mg.	*mg.*	*per cent*
Blood	60.0	56.6	3.4
Kidneys	92.4	91.2	1.2
Liver	96.8	90.1	6.7
Spleen	93.2	92.2	1.0
Muscle	88.4	88.4	0.0

TABLE X.

Dog Killed by Chloroform 1 Hour after Injection of Phenol.

Organ.	Total phenols per 1,000 gm.	Free phenols per 1,000 gm.	Conjugated phenols.
	mg.	*mg.*	*per cent*
Blood	50.0	33.3	16.7
Kidneys	117.6	100.0	17.6
Liver	117.6	100.0	17.6
Spleen	108.6	92.4	16.2

Baumann found nineteen times more conjugated phenol in the liver than in the blood of a dog poisoned with phenol. One of our dogs succumbed to an overdose of phenol given by stomach and we were able to confirm Baumann's observation. It is probable that in cases of fatal phenol poisoning by stomach the phenol

absorbed from the intestinal tract is temporarily held by the liver and that there is not sufficient time nor proper circulation before the death of the animal for complete distribution to the tissues. Table IX shows that no such concentration of phenols in the liver occurs when the fatal poisoning is caused by injection into the jugular vein.

DISCUSSION.

We may review our conception of phenol metabolism somewhat as follows:

A small part of the tyrosine of the food proteins is broken down by bacterial action into hydroxy-acids such as *p*-oxyphenylproprionic, *p*-oxyphenylacetic, and *p*-oxybenzoic acids, and into volatile phenols, primarily *p*-cresol and phenol. The hydroxy-acids which have no toxic effects are not subjected in any noticeable degree to oxidation or conjugation and are practically completely excreted in the urine in the free state. The volatile phenols which are very toxic even in small amounts are dealt with in an entirely different way. More than half of these toxic phenols are oxidized by the intestinal mucosa, body fluids, and liver parenchyma. The remainder is conjugated in the liver with sulfuric or glucuronic acids. After passing from the liver the conjugated phenols are uniformly distributed to all the tissues and are rapidly eliminated by normal kidneys probably within 12 hours. From the experiments given in the following paper we feel confident that the synthesis of phenol-sulfuric and phenol-glucuronic acids takes place only in the hepatic parenchyma.

SUMMARY.

Intravenous injection of phenols is followed by a prompt and uniform distribution of such substances in the body fluids and tissues.

Following such injection we note a rapid disappearance of free phenols from the blood and a rapid increase in conjugated phenols. This rise in conjugated phenols usually reaches a maximum within 1 hour and thereafter slowly declines with excretion. We note too a uniform distribution of these conjugated phenols throughout the body fluids and tissues.

Ingestion of phenols gives a totally different picture. With a sufficiently large dose some free phenols appear in the blood for a short period—rarely more than 30 minutes. The conjugated phenols show a maximum rise during the first and second hours and subsequent decrease due to renal elimination.

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STUDIES OF LIVER FUNCTION.

III. PHENOL CONJUGATION AS INFLUENCED BY LIVER INJURY AND INSUFFICIENCY.

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In a recent article dealing with the study of liver function Delprat and Whipple (2) review some of the difficulties arising in this work. We refer to that paper for a discussion of the many factors which complicate a critical study of hepatic function, liver injury and repair, together with the great reserve capacity of the liver cells. The ideal liver function test is not at hand and any studies in this difficult field should be of interest alike to the physiologist and the physician. We must keep in mind that any satisfactory *liver function test must include some factor of strain or load* to determine the upper limits as well as the lower levels of liver function.

EXPERIMENTAL OBSERVATIONS.

Tables XI to XVI give the results of some of the experiments we have carried out. The general plan of these experiments was as follows: the dog was first standardized, either after several days of fasting or several days of carbohydrate diet in order to determine the time required by the liver to conjugate the standard dose of *p*-cresol. Liver injury was then produced by one of a number of methods and another test was made to observe the capacity of the injured liver. Tables XI and XIV are given as representative of over a score of standardization experiments on carbohydrate diet or fasting. All of them show surprising uniformity. The percentage conjugation of added phenols and the time required to do this work give a fairly accurate idea of

the ability of the liver to conjugate phenols. In the calculation of the *percentage conjugation* of *added phenols* the assumption is made that the free and total phenols present before ingestion remain constant during the experiment and are not influenced by any of the procedures such as the repeated bleeding or the ingestion of fluid, etc. That this is actually the case is shown in Table XIV where one of three control experiments is reported. These experiments were similar in every way to the other tests performed, except that, instead of the phenol solution, a corresponding amount of water was given by stomach tube.

It may be advisable to explain by example how the *percentage conjugation* of added phenols is calculated:

	Total phenols.	Free phenols.
	mg.	*mg.*
Before ingestion	24	22
After "	28	24

The increase of total phenols after ingestion over total phenols before ingestion is 4 mg. The increase of free phenols after ingestion over free phenols before ingestion is 2 mg. The difference between the two, 2 mg. (or 50 per cent) of the added phenols, has been conjugated. The percentage conjugation of added phenols at any given time after ingestion may, therefore, be calculated by the following simple algebraic formula.

$$\frac{a - b}{c - d} .100 = \text{percentage conjugation}$$

where a represents free phenols after ingestion, b free phenols before ingestion, c total phenols after ingestion, d total phenols before ingestion. In the case above

$$\frac{24 - 22}{28 - 24} .100 = 50 \text{ per cent}$$

We have used the method of Davis and Whipple (1) to produce standard liver injury and necrosis with consequent impairment of function. All operative procedures were done under surgical ether anesthesia.

TABLE XI.

Chloroform Injury and Eck Fistula Experiments.

Dog 21-100. White bull, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Mar. 25. Standardization on carbohydrate diet.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	23.1	22.2	3.9	
After "				
10 minutes	26.7	24.0	10.0	50.0
20 "	29.4	23.8	19.0	75.0
30 "	29.4	22.5	27.0	95.0
1 hour	33.3	22.2	33.3	100.0
2 hours	30.0	21.1	29.7	
Apr. 16. Chloroform anesthesia (1¼ hrs.). Apr. 18, *p*-cresol ingestion.				
Before ingestion	49.3	48.1	2.4	
After "				
10 minutes	53.5	52.1	2.6	4.8
20 "	54.9	50.5	8.0	57.0
30 "	55.6	50.5	9.2	62.0
1 hour	54.1	47.6	12.0	100.0
2 hours	59.4	54.1	8.9	
Apr. 21. Eck fistula operation. Apr. 27, *p*-cresol ingestion.				
Before ingestion	21.1	20.7	1.4	
After "				
10 minutes	25.0	24.4	2.4	5.1
20 "	28.4	26.7	6.0	17.8
30 "	30.0	27.0	10.0	29.2
1 hour	30.7	26.7	13.2	37.4
2½ hours	30.0	24.7	17.7	55.0
5½ "	25.1	22.4	10.8	57.5
May 13. *p*-Cresol ingestion, conjugation much decreased.				
Before ingestion	35.0	34.0	2.8	
After "				
10 minutes	37.8	36.2	4.2	21.4
20 "	39.4	37.0	6.1	31.8
30 "	39.4	35.7	9.4	61.3
1 hour	42.0	40.0	5.0	14.3
2 hours	41.0	39.0	4.9	16.6

TABLE XI—*Concluded.*

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
May 20. Ligation of hepatic artery. May 24, *p*-cresol ingestion.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	27.0	25.0	7.4	
After "				
15 minutes	35.7	33.7	5.6	0.0
30 "	41.5	39.0	6.0	3.4
1 hour	45.4	42.6	6.2	4.3
2 hours	47.6	45.2	5.0	2.0
4½ "	40.8	36.7	10.0	15.2
May 25. Death. Hepatic insufficiency				

Experimental Protocol of Dog 21–100 (See Table XI).

Mar. 25, 33 lbs., healthy. Standardized with 1,650 mg. of *p*-cresol after 4 days carbohydrate diet. No reaction. Apr. 16, 75 minutes chloroform after 4 days fasting. Apr. 18, 35 lbs., 1,750 mg. of *p*-cresol, no reaction. Conjugation about one-half normal. Apr. 21, Eck fistula operation. External surface of liver shows that the chloroform injury has not been completely repaired—the centers of the lobules are hyperemic and stand out distinctly from the opaque periphery. Apr. 27, 30 lbs., 1,500 mg. of *p*-cresol. ½ hour after ingestion the dog is severely intoxicated. Conjugation about one-third normal. Operative wound is partly open and infected. May 1 wound sewed up. May 3, wound again open and sewed up. May 13, slight muscle tremors—prominent distension of abdominal cutaneous veins. 32 lbs., 1,600 mg. of *p*-cresol. No reaction, but very weak. Conjugation about one-third normal. May 20, no tremors, no ascites, collateral circulation less prominent than on May 13. 30 lbs. Arch of hepatic artery tied in two places at 11 a.m. 1,500 mg. of *p*-cresol by stomach tube immediately after operation. Very severe reaction which may, in part, have been due to the ether. Temperature at 1 p.m. 36.2°; at 2 p.m. 37.2°; at 3 p.m. 37.9°. Conjugation one-fourth normal. May 24, very weak and slight muscle tremors. Hematocrit red cells 38 plus. 25 lbs. 1,250 mg. of *p*-cresol, severe reaction. Conjugation less than one-fourth normal. May 25, died 5 days after ligation of hepatic artery.

Autopsy.—May 25. Thorax, heart, and lungs normal. Spleen fibrous and rather pale. Serous surfaces clean except plastic adhesions and yellowish fibrinous exudate about site of hepatic artery operation. Stomach and intestines not abnormal. Kidney and pancreas negative. Hepatic artery ligated in two places and completely occluded.

Eck fistula about 6 mm. long and clean. There must have been considerable flow through this opening or it would have closed. The ligature

above the Eck fistula on the portal vein did not completely occlude the lumen. Lumen probably about 1 mm. in diameter. The knot must have slipped before the second tie was made.

Liver is atrophic but not exactly like the usual Eck fistula specimen. It is not as translucent. The lobules are small and brown at the margins—yellow in the centers, probably necrotic. Some areas are swollen, yellow, and opaque. The lobules here are larger and yellow—necrosis probable. These areas are not numerous, but pretty sizable, $2 \times 2 \times 5$ or 6 cm. for the largest—the volume is estimated as about one-tenth or less of the liver parenchyma.

Microscopic Examination.—Spleen, much pigment and phagocytes. Liver, some sections show extensive hyaline necrosis, involving the centers of the lobules up to 90 per cent in extent—few liver cells escape at the margins of the lobules. Other sections show central atrophy alone (usual Eck picture) with phagocytes full of lipochrome pigment. Others show some evidence of repair, enlarged liver cells, mitoses, etc. Other organs of no importance.

Most instructive are experiments performed on two Eck fistula dogs.

Standardized on Mar. 25, Dog 21-100 showed 95 per cent conjugation within 30 minutes (Table XI). On Apr. 16 a liver injury of about 60 or 70 per cent was produced by chloroform. Table XI, experiment on Apr. 18, shows slightly less than 50 per cent reduction of function. On Apr. 21 the Eck fistula operation was performed. On Apr. 27 the test with *p*-cresol revealed a one-third normal conjugation. A repetition of this test on May 13 shows a slight improvement in function. The arch of the hepatic artery was ligated in two places on May 20 and on testing with *p*-cresol we found that there remained less than 3 per cent of the original capacity of the liver to conjugate phenols. Death of the dog was due to liver insufficiency.

The story of the other Eck fistula dog is similar.

Table XII shows that Dog 21-105 exhibited perfectly normal conjugation when standardized on a carbohydrate diet. On Apr. 5 we performed an Eck fistula operation and a conjugation test on Apr. 12. It appears that only 27.8 per cent of the added phenols had been conjugated 2 hours after ingestion, whereas before the operation 100 per cent conjugation occurred in 20 minutes—the function of the liver on Apr. 12 we may estimate as about 10 per cent of normal. On Apr. 20 another test was made and some improvement noted. The conjugation at this time amounted to about 25 per cent of normal. On May 16 we ligated the hepatic artery in one place, leaving some of the collaterals to the liver patent. It will be seen that the liver function after this operation amounted to somewhat more than 5 per cent of normal.

TABLE XII.

Eck Fistula Experiments.

Dog 21-105. Black, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Mar. 31. Standardization on carbohydrate diet.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	24.3	23.4	3.7	
After "				
10 minutes	26.0	23.5	9.6	94.0
20 "	28.2	22.0	22.0	100.0
30 "	29.0	22.5	22.4	100.0
1 hour	35.0	21.8	37.7	100.0
2 hours	33.0	23.0	30.0	100.0
Apr. 5. Eck fistula operation. Apr. 12, *p*-cresol ingestion.				
Before ingestion	25.5	24.7	3.1	
After "				
10 minutes	33.1	31.2	5.7	14.4
20 "	34.2	32.2	5.8	13.8
30 "	36.8	34.5	6.2	13.2
1 hour	37.7	34.5	8.5	19.6
2 hours	36.3	32.5	10.4	27.8
Apr. 20. 15 days after Eck fistula operation.				
Before ingestion	27.0	26.7	1.1	
After "				
10 minutes	38.0	36.2	4.7	13.6
20 "	38.0	35.1	7.6	23.6
30 "	42.5	38.5	9.4	23.9
1 hour	42.0	35.7	15.0	40.0
2 hours	37.0	30.0	18.9	67.0
3 "	33.5	25.0	25.3	100.0
May. 16. Immediately after partial ligation of hepatic artery. Conjugation greatly decreased.				
Before ingestion	26.7	26.0	2.6	
After "				
15 minutes	43.5	41.7	4.1	6.5
30 "	47.0	44.7	4.8	7.8
1 hour	52.5	48.1	8.3	14.3
$1\frac{1}{2}$ hours	52.0	46.9	9.8	17.3
$2\frac{1}{2}$ "	40.9	35.0	14.4	36.6
5 "	37.0	30.2	18.3	59.2
May 18. Death. Hepatic insufficiency and peritonitis.				

Experimental Protocol of Dog 21–105 (See Table XII).

Mar. 27, carbohydrate diet begun. Mar. 31, 29 lbs., slight distemper. Standardized with 1,450 mg. of *p*-cresol. No reaction. Apr. 5, Eck fistula operation. No food. Apr. 6, water and bread. Apr. 7, regulation diet: carbohydrates, vegetables, kaolin, and bones. No meat or milk. Apr. 12, 26 lbs., slight distemper. Moderate ascites. 1,300 mg. of *p*-cresol by stomach tube. Moderately severe reaction. ½ hour after ingestion dog falls down occasionally when trying to walk. 1 hour after dog does not fall down but is still very shaky. Phenol conjugation is about one-tenth normal. Apr. 20, 30 lbs., no distemper. Severe ascites. Collateral circulation on abdominal wall is prominent. 1,500 mg. of *p*-cresol by stomach tube. Moderate reaction. A half hour after ingestion the dog sways from side to side when trying to walk but does not fall. Phenol conjugation about one-fifth normal (a little better than on Apr. 12). May 16, 26 lbs., moderate ascites. Ligature on hepatic artery in only one place, 1,300 mg. of *p*-cresol. Moderate reaction. Dog has apparently recovered late in afternoon. Phenol conjugation less than one-tenth normal. Hematocrit red cell 42 per cent. May 18, dog died 32 to 38 hours after last operation.

Autopsy.—May 18. Ascitic fluid less than at operation—now about 100 cc. The fluid is turbid and the serous surfaces are injected—there is a perforation in the first third of the duodenum and shortly before death there evidently had been an escape of intestinal contents with recent peritonitis. Thorax, heart, and lungs negative. Blood clots normal. Spleen small and fibrous; not much blood. Adhesions about the site of the first operation are numerous and bled easily at the second operation. Liver small, decidedly yellow, due to fat. Lobules show much injury (fat) and perhaps necrosis—there is no edema and the veins are clear. Fistula is about 5 mm. in length and is less than one-half its original length. The edges are smooth and there is no thrombosis. The passage of blood through it was obviously difficult and caused the portal stasis, development of collaterals, and ascites. Intestinal tract negative in general. Duodenum shows a sharp punched out area about 3 × 1 cm. due probably to ligature of the hepatic artery. Kidneys negative.

Microscopic Examination.—Much fat in liver cells (large and small droplets), central necrosis is abundant—about one-third of cell lobules. Kidneys negative. Death explained by liver injury plus terminal duodenal perforation.

Chloroform poisoning gives considerable information as to the conjugation of phenols in the liver. It also points out the fact that *this method is inadequate to measure the high limits of liver capacity.* For example, given a chloroform injury of approximately one-third of the liver parenchyma we may expect a normal or almost normal conjugation of phenols after administration of

the unit dose with the usual routine technique. This means that our test does not reveal the maximum capacity of the normal liver in conjugation of phenols. When we have a chloroform liver injury of one-half to two-thirds of the liver lobule we can then demonstrate a considerable impairment of liver conjugation (compare Table XI, Experiment of April 18). With more advanced liver injury we note a great drop in liver conjugation (compare Table XIV, Experiment of April 22). Finally with a *lethal chloroform injury* involving the greater part (90 per cent or more) of each liver lobule (Table XIII, Dog 21–113) we observe *zero liver conjugation.*

Phosphorus injury gives a similar picture of impaired liver function (Table XIII). It is more difficult to estimate the amount of liver injury in phosphorus poisoning as cell necrosis is not a conspicuous feature of the injury. We note two experiments in Table XIII to show liver function impairment. The injury was fatal in one experiment and very severe in the second animal, yet there was a certain amount of phenol conjugation in both experiments.

We observed no impairment of liver function as shown by phenol conjugation in bile fistula dogs. It is interesting to recall that one liver function test (phenoltetrachlorphthalein) shows a distinct fall in output in bile fistula dogs indicating that the chronic cholangitis which is usually present in such animals interferes with the elimination of phenoltetrachlorphthalein but not with the conjugation of phenols.

Another important control is given by dogs sick with *distemper,* as it might be argued that intoxication of any sort might disturb this conjugation of phenols. One animal (Table XVI) was profoundly prostrated with acute distemper and died shortly after the completion of the experiment yet a normal conjugation followed the administration of the unit dose of *p*-cresol. Autopsy showed the familiar lesions of acute distemper and nothing else. Under these conditions therefore the capacity of the liver to conjugate phenols is in nowise impaired.

From an examination of Table XI, Experiment of April 18, it will be seen that following chloroform injury the initial concentration of total phenolic substances in the blood is more than twice, and, in some cases (fatal chloroform injury, Table XIII),

TABLE XIII.

Chloroform and Phosphorus Injury of Liver.

Dog 21-113. Mongrel, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.

May 2. Chloroform anesthesia, $1\frac{1}{2}$ hours. Liver necrosis almost total. Conjugation zero.

May 3. *p*-Cresol by ingestion.

	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	103.0	99.2	3.0	
After "				
10 minutes	111.0	107.0	3.6	2.5
20 "	121.5	117.0	3.7	3.8
30 "	124.0	121.0	2.4	0.0
1 hour	135.0	131.0	3.0	0.6
2 hours	133.0	130.0	2.3	0.0

Dog died 2 days after injury. Microscopic examination shows only 1 to 2 rows of surviving liver cells about portal veins.

Dog 21-112. Black, male.

Phosphorus injury, fatal. Conjugation impaired.

Before ingestion	42.5	42.0	1.2	
After "				
10 minutes	49.5	47.0	5.5	28.5
20 "	51.5	47.6	7.6	37.7
30 "	52.6	46.0	12.5	60.4
1 hour	60.5	50.0	17.3	55.5
$2\frac{1}{2}$ hours	58.5	38.5	34.2	100.0

2 mg. of phosphorus per pound body weight, in oil subcutaneously. Test made 1 day after injury. Dog died 36 hours after injection. Autopsy shows fatty degeneration of liver.

Dog 21-76. Mongrel, male.

Phosphorus injury, sublethal but severe. Conjugation impaired.

Before ingestion	26.7	26.0	2.7	
After "				
10 minutes	32.6	27.0	17.1	83.0
20 "	35.6	29.2	17.7	61.3
30 "	40.8	31.5	22.7	61.0
1 hour	40.0	28.2	29.5	83.5
2 hours	34.2	25.2	26.3	100.0

2 mg. of phosphorus per pound body weight, in oil subcutaneously. Test made 2 days after injury. Dog recovered very slowly.

four times as high as in the blood of fasting normal dogs or dogs on a carbohydrate diet. We have no definite knowledge regarding all the factors responsible for this pronounced increase, but it is

TABLE XIV.

Chloroform Liver Injury and Controls.

Dog 21-80. Brown, female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Apr. 15. Standardization after 4 days fasting.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	30.8	29.4	4.5	
After "				
10 minutes	35.2	30.2	14.2	98.0
20 "	37.8	30.2	20.1	89.0
30 "	38.5	28.0	27.3	100.0
1 hour	40.0	28.5	28.7	100.0
2 hours	36.4	28.2	22.5	100.0
Apr. 20. Chloroform anesthesia 1 hour, 20 minutes. Apr. 22, *p*-cresol by ingestion.				
Before ingestion	31.3	30.0	4.1	
After "				
10 minutes	43.5	41.0	5.7	9.8
20 "	48,5	43.6	10.1	20.9
30 "	47.8	41.8	12.5	28.4
1 hour	46.5	40.0	14.0	34.2
2 hours	40.0	33.0	17.5	65.2
Effect of repeated bleedings. Apr. 28. Water given instead of phenol solution.				
Before ingestion	27.2	26.8	1.4	
After "				
10 minutes	27.0	26.9	0.4	
20 "	26.8	26.8	0.0	
30 "	27.5	27.2	1.0	
1 hour	26.9	26.7	0.7	
2 hours	26.5	26.4	0.4	

interesting to speculate about some of them. It has been shown by Dubin (3) that in a fasting dog the concentration of urinary phenols, after an initial drop rises considerably. In our cases

TABLE XV.

Bile Fistula Control.

Bile fistula dog (old white bull, female).

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	22.7	21.5	5.3	
After "				
10 minutes	31.2	28.3	9.3	20
30 "	34.5	22.7	34.2	89
1 hour	31.1	22.7	27.0	86
2 hours	30.8	20.8	32.4	100

Bile fistula of over 2 years duration—dog normal.

TABLE XVI.

Acute Distemper Intoxication—Control.

Dog 21-98. Black-brown, long haired female.

Time.	Total phenols per 1,000 cc.	Free phenols per 1,000 cc.	Conjugated phenols in percentage of total.	Conjugation of added phenols.
Mar. 23. Standardization.				
	mg.	*mg.*	*per cent*	*per cent*
Before ingestion	28.3	27.8	1.7	
After "				
10 minutes	37.0	33.3	10.0	36.5
20 "	43.1	37.4	13.2	34.7
30 "	47.1	40.0	15.0	35.1
1 hour	41.7	28.9	30.7	91.7
2 hours	37.7	25.4	32.6	100.0
Apr. 13. Terminal distemper.				
Before ingestion	29.0	28.4	2.0	
After "				
10 minutes	38.0	33.0	13.1	48.9
20 "	42.4	37.0	12.7	36.0
30 "	45.2	38.3	15.2	38.9
1 hour	40.0	29.8	25.5	87.2

Very severe distemper, toxic condition, slight reaction to cresol after 10 minutes, from which dog completely recovered in 30 minutes. Died of distemper after 1 hour. Postmortem: No pneumonia.

of chloroform injury the period of fasting (3 to 4 days) was certainly not long enough to account for the tremendous increase; this is further controlled by the normal fasting dogs (Table XIV and others) in which no such increase occurs. It has been pointed out that the increase of urinary phenols in prolonged fasting is due to a great destruction of proteins. This cell destruction may be one of the factors producing the rise of blood phenols in liver injury. As mentioned before, proteins and their decomposition products such as tyrosine, tryptophane, and other easily oxidizable substances give the blue color with the phenol reagent, and since the increase of conjugated, volatile phenols in these cases is not proportional to the increase in total phenols (the conjugated phenols being practically the same as in normal dogs) the bulk of the additional color-producing bodies may be proteins and their decomposition products other than phenols, or sugars and related substances (Gortner and Holm, 4; Levine, 5).

It was shown in the preceding paper that a large part of the absorbed phenols is destroyed by a process of oxidation entirely different from the synthetic process by which conjugated phenols are produced. It is possible that this oxidative destruction which goes on in most tissues, but to greatest extent in the liver and in the intestinal epithelium, is inhibited or at least greatly reduced by the presence of injured liver tissue, thus adding to the increase in phenolic substances. This seems to be substantiated by the fact that in dogs injured by chloroform the absolute rise in blood phenols after ingestion of *p*-cresol is always greater than in normal dogs. Table XI, Experiment of April 18, and Table XIII, Dog 21–113 show further that with chloroform injury the excretion of ingested phenols may be less rapid than in normal dogs—another less important factor in the increased concentration of phenolic substances in the blood. Delprat and Whipple (2) showed that there is no impairment of *renal* function following a chloroform anesthesia as measured by the elimination of phenolsulfonephthalein.

In accounting for this increased concentration of "phenolic reacting substances" in blood in cases of chloroform injury, we have, in addition to whatever unknown factors may play a part, these possibilities: (1) an increased destruction of body proteins, particularly liver proteins, by chloroform; (2) an inhibition or

lessening of the oxidative destruction of absorbed phenolic substances; and (3) a slowing of the excretion of phenolic substances.

It is interesting to note that this rise in blood "phenols" seldom occurs in dogs whose liver has been injured with phosphorus. Even fatal phosphorus injury shows only a slight increase over the normal level (Table XIII). Nor does this increase occur with bile fistula or Eck fistula dogs. This indicates again the presence of certain "phenol-reacting" substances in the blood of dogs poisoned with chloroform but not necessarily true phenols alone.

DISCUSSION.

The cause of death in Eck fistula dogs has long been a puzzle to physiologists. It has been claimed by many workers that this peculiar intoxication was due to the absorption of the toxic amino-acids as it is well known that heavy meat feeding will precipitate the characteristic intoxication. Unpublished experiments of Van Slyke and Whipple show that there is no abnormal heaping up of amino nitrogen in the blood during periods of meat feeding in Eck fistula dogs. No amino-acids, such as have been noted in severe cases of chloroform poisoning and fatal liver injury, appear in the urine of these Eck fistula dogs. The experiments given in this paper indicate clearly that the Eck fistula liver is incapable of normal conjugation of one toxic radicle, (*p*-cresol). This disability is noted during periods of normal health as indicated by clinically normal reactions. It is at least possible that this impairment of the conjugating powers of the liver is responsible for the toxic developments in the Eck fistula dog. We note that there is no heaping up in the blood of the Eck fistula of any phenol-reacting substances. It will be of considerable interest to study this reaction in the Eck fistula dogs on a high meat diet and during periods of the characteristic Eck fistula intoxication. It is to be recalled that these Eck fistula dogs were maintained on a diet of rice, bread, milk, and bones.

It is significant to note in the tables that severe poisoning with phosphorus will not cause a great rise in the total blood phenols but equally severe poisoning with chloroform will give very high figures for total blood phenols. One suspects that cell necrosis which is so conspicuous in chloroform poisoning is responsible for this difference. This reasoning suggests that a considerable

part of the total phenol of the blood in chloroform poisoning may be due to "*phenol-reacting substances*" quite apart from the phenolic substances.

The method we have used is of interest to investigators but as yet of little practical value to internists. *p*-Cresol is too toxic to be used clinically but further study may enable us to suggest some non-toxic radicle which will test these synthetic activities of the liver parenchyma. It is of some significance that this method is a specific test of liver function, as we have evidence that other body cells are not concerned in the conjugation of phenols.

SUMMARY.

The conjugation of phenols in the body is in nowise disturbed by bleeding periods, by the presence of a bile fistula nor by a lethal intoxication (distemper). Under the conditions of these experiments the reaction to ingestion of a unit dose of *p*-cresol is uniform and associated with a constant amount of phenol conjugation which can be measured quantitatively.

The presence of an Eck fistula modifies this reaction and *reduces* the amount and speed of phenol conjugation. At times the Eck fistula liver function of conjugation may fall to one-third or even to one-tenth of the normal. When the liver circulation is further impaired by partial ligation of the hepatic artery in an Eck fistula dog, we may observe a fall in phenol conjugation to 3 or 5 per cent of normal. Liver exclusion, therefore, will eliminate phenol conjugation.

The presence of a slight liver injury due to chloroform or phosphorus may not modify the phenol conjugation. Extensive liver injury due to these poisons will always lessen phenol conjugation. Extreme and *fatal liver injury* (chloroform) *will reduce phenol conjugation to zero.*

These observations lead us to conclude that *phenol conjugation* is a function of *liver parenchyma cells* and of no other body cells.

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THE EFFECT OF HYDROGEN ION CONCENTRATION UPON THE DETERMINATION OF CALCIUM.

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McCrudden's (1, 2) method has come to be recognized as the best for the accurate determination of calcium. The method is to precipitate the calcium as oxalate in the presence of sufficient ammonium chloride to hold the magnesium oxalate in solution, and of sufficient acid to hold the calcium oxalate partly in solution. Sodium acetate is then added to decrease the acidity and to precipitate the rest of the calcium oxalate on the crystals already formed. Thus, large crystals are made which are easy to filter and are not contaminated with occluded magnesium or calcium phosphate.[1] The amount of acetate is selected so as to give a solution not acid enough to dissolve the calcium oxalate, nor alkaline enough, if cold, to allow calcium phosphate to precipitate. These directions are empiric and must be followed accurately, as the author cautions.[2] Just what determines the amount of acetate is not definite, for he states that in the ash of feces 15 instead of 8 cc. are required.[3]

The sodium acetate added regulates the acidity of the solution. Unfortunately, at the time McCrudden wrote his article, Sörensen's (3), Michaelis' (4), and Clark's (5) monographs on hydrogen ion concentration were not published. In 1913, Hildebrand (6) of the Bureau of Standards showed the necessity of a systematic investigation of analytical methods with regard to the hydrogen ion concentration. Such information is not as yet available for

[1] McCrudden (1), p. 99.
[2] McCrudden (1), p. 100.
[3] McCrudden (2), p. 198.

many types of analysis. Except that of Kramer and Tisdall (7) there is none for calcium. We have, therefore, critically examined McCrudden's method in relation to hydrogen ion concentration.

Theory Underlying the Precipitation of Calcium as Oxalate.

The determination of calcium in the presence of magnesium and phosphate depends primarily upon the solubility product of the various precipitates involved; secondarily, upon the hydrogen ion concentration.[4] As a development of the last 30 years in the chemistry of solutions, the theory of ionization has been applied to the problem of precipitation of nearly insoluble substances with great success.

Solubility Product.— Stieglitz (8) and Noyes (9) have presented very carefully in their manuals of qualitative analysis the relation between ionization and precipitation. The basic principle is the mass law, which can be stated.[5]

$$\frac{(A^+) \times (B^-)}{(A\ B)} = K$$

In non-mathematical terms, when a substance is present in water an equilibrium is established between the concentration of the undissociated salt in solution and the concentration of the ions. The value, K, is a constant depending upon the nature of the particular salt. Or, when there are two ions present which form an insoluble compound, precipitation occurs until the product of the concentration of the ions divided by the concentration of undissociated salt is a constant which is the K for that salt.

A simpler relation which holds with sufficient accuracy for practical purposes is that the product of the ions is a constant. This constant is known as the "solubility product."

$$(A^+) \times (B^-) = K$$

Obviously, we decrease the amount of either ion present by increasing the other ion. If we double A^+ we halve B^-. Hence, adding more A^+ causes more and more complete precipitation

[4] The question of the problem of occlusion and the conditions for obtaining large crystals have been discussed by McCrudden (1), p. 99.

[5] Parentheses about a symbol mean concentration. Thus, H^+ means hydrogen ion; (H^+) means hydrogen ion concentration.

of B^-. Thus, one adds an excess of oxalate ions, so that the calcium is more completely precipitated. The value of the solubility constant is very important for it determines which salt will precipitate. The salt having the smaller solubility product will precipitate and the other will remain in solution. If all the factors are known a quantitative expression can be calculated rigorously from the mass law. The solubility product determines whether calcium will be precipitated as a phosphate or carbonate or oxalate in solutions containing these acids. The solubility products of the various salts to be considered are given in Table I.

Effect of Hydrogen Ion Concentrations.—Acids on dissociation always give hydrogen ions, H^+. The extent of the acidity depends upon the concentration of the hydrogen ions, (H^+) (10). The effect of hydrogen ions in precipitation can best be discussed under three heads: (*a*) the effect on basic salts, (*b*) the change in the ionization constant of the acid radicals, and (*c*) the suppression of ionization of weak acids.

The effect of acid on basic salts is to depress the ionization of the hydroxyl ions; for substances in solution are related to the ionization of water. Water itself gives H^+ ions and also OH^- or basic ions. The relation of the ions is expressed by the mass law:

$$\frac{(H^+) \times (OH^-)}{(H_2O)} = 10^{-14}$$

When one adds H^+ ions to water one depresses the ionization of the hydroxyl ions since the product of these ions is a constant. Thus, in the case of magnesium hydroxide, by adding acid one decreases $(\overline{O}H)$ to such an extent that in acid solution its solubility product is never reached and hence no magnesium hydroxide can precipitate.

By adding acid one changes the ionization constant of the acid. Thus, in the case of the tri-basic phosphate the addition of H^+ ions gives the following reaction:

$$\overline{\overline{\overline{PO}}}_4 + H^+ = \overline{\overline{HPO}}_4 + H^+ = H_2\overline{PO}_4$$

The tri-basic salt can only exist in alkaline solution and as one increases the hydrogen ion concentration the di-basic and monobasic salt must be formed. Thus, in an acid system there is no calcium phosphate but only the more soluble di-basic and mono-

basic calcium phosphates. The solution, if quite acid, about pH 3.0, will have the same effect on calcium oxalate.

$$H^+ + (\bar{C}_2\bar{O}_4) \rightleftharpoons (H\bar{C}_2O_4)$$

By adding acid to a solution containing ions of a weak acid one converts the highly ionized salt into a slightly ionized acid, according to the equation:

$$2H^+ + (\bar{C}_2\bar{O}_4) \rightleftharpoons H_2C_2O_4$$

Thus one removes oxalate ions from the solution by adding acid. If the ionization is repressed below the solubility product no precipitate is formed.

Each of these factors: the effect on basic salts, the change in the ionization constant of the acid, and the suppression of ionization, influence the precipitation. Therefore, we shall consider each of the salts that may be formed in the course of the analysis of calcium in the presence of magnesium and phosphates, in an acid solution of pH 4.0 to 6.2.

Calcium Oxalate and Magnesium Oxalate.—These salts are precipitated in the presence of an excess of oxalate ions. The question is whether there is any danger of precipitating magnesium with the calcium. This has been discussed in part by Kramer and Tisdall (7). The solubility product of these salts is sufficiently different so that Gooch (11) recommends that even in the presence of ten times the amount of magnesium it is not necessary to carry out double precipitation. In acid solution this difference in solubility product is even more marked.

Acid added to a solution of calcium and magnesium oxalates favors the precipitation of the calcium in two ways. It forms the acid salts which are more soluble. The acid salt of calcium oxalate, at this acidity (pH 4.0 to 6.2) is not formed in sufficient amount to have any appreciable effect on the solubility. No study of the magnesium salts has been made, but from general evidence it is concluded that this would be more readily affected by acids and hence more soluble.

Second, the addition of acid suppresses ionization, in this case from about 40 to about 30 per cent. The diminished ioniza-

tion affects the calcium oxalate much less than the magnesium oxalate as the former has a much smaller solubility product. Any acidity not great enough to form acid calcium oxalate will be a factor in preventing the formation of magnesium oxalate and in favoring the precipitation of calcium oxalate.

Magnesium Hydroxide and Magnesium Ammonium Phosphate.—Both salts are extremely insoluble but since they do not occur in acid solutions they do not precipitate. Hildebrand (6) says that the former precipitates at pH 7.4 to 8.0 and that the latter first appears at pH 6.6.

Mono-, Di-, and Tri-Basic Calcium Phosphate.—The salts of calcium form a very difficult problem since as found by Cameron (12) and his collaborators and many others, these salts vary in their composition according to the source, are decomposed by water, and take months to come to equilibrium.

For orientation we attempted to find the pH of saturated solutions of the mono-, di-, and tri-basic salts of calcium phosphate. These salts (Baker's analyzed) were washed with distilled water. The acidity of the supernatant liquids, determined by the colorimetric method (5), became roughly constant at room temperature in 24 hours and gave the following values:

$$CaH_4(PO_4)_2 = \text{pH } 5.0$$
$$CaHPO_4 = \text{pH } 7.4$$
$$Ca_3(PO_4)_2 = \text{pH } 6.8$$

We found that no calcium solution more acid than pH 4.0 which contained phosphates yielded a precipitate after boiling, but Patten and Mains (13) report a precipitate at pH 2.3 at 26°. But the more acid the point at which these salts precipitate, the greater is the proportion of phosphate to calcium. And, as one can see by glancing at Table I, the more acid the salt the greater is the solubility product. Therefore, at pH 4.0 to 6.4 there is no danger of any calcium being present as phosphates. At this acidity the calcium phosphates have a solubility product more than a million times as great as calcium oxalate. Breazeale (14) remarks it is quite safe to precipitate calcium phosphate quantitatively as calcium oxalate by adding oxalic acid. Therefore, there is no danger of the precipitation of calcium acid

phosphate if the precipitation of calcium oxalate is carried out at the proper acidity.

TABLE I.

*Data on Solubilities.**

Salt.	Per liter.	Mols per liter.	Solubility product.
	gm.		
CaC_2O_4	0.0055	0.000044	1.9×10^{-9}
$Mg(OH)_2$	0.009	0.00015	3.5×10^{-12}
$MgNH_4PO_4$	0.05	0.0068	3.0×10^{-7}
MgC_2O_4	0.302	0.0027	4.8×10^{-5}
$Ca_3(PO_4)_2$	0.01	0.00033	2.8×10^{-18}
$CaHPO_4$	0.2	0.00147	2.0×10^{-8}
$CaH_4(PO_4)_2$	18.0	0.77	4.0×10^{-1}

* The figures are compiled from Landolt-Börnstein, Physikalisch-chemische Tabellen; A. Seidell, The solubilities of inorganic substances; The Chemische Kalender, 1914—Dictionary of Solubilities, etc.

EXPERIMENTAL APPLICATION OF THE THEORY.

It is important to verify, first, the most acid limit of acidity; second, the least acid limit; and third, the best method of obtaining the desired acidity.

Most Acid Limit.—The most acid limit is the point at which calcium oxalate begins to be converted into the more soluble acid calcium oxalate. McCrudden has determined the amount of sodium acetate, which, under the conditions of his procedure will prevent the solution of calcium oxalate.[6] We determined colorimetrically the hydrogen ion concentration of solutions precipitated according to his directions. In all solutions more acid than pH 4.4, which contain less than 6 cc. of sodium acetate, the results are low. Further experiments in Table II show that correct determinations are made at pH 4.0, which is, therefore, the most acid limit for the determination of calcium oxalate.

The Least Acid Limit.—The least acid limit is the point at which magnesium ammonium phosphate and magnesium hydroxide precipitate. According to Hildebrand this is pH 6.6 to 7.6. To fix this point experimentally we carried out the precipitation

[6] McCrudden (1), p. 90.

TABLE II.

*The Amount of Sodium Acetate and the Resulting Acidity.**

No.	20 per cent sodium acetate.	pH calculated.	pH determined.	Calcium.
	cc.			*mg.*
1	0		1.3	30.1
2	4	2.8	2.8	32.0
3	5	4.0	4.0	33.1
4	6	4.4	4.4	33.1
5	10	4.8	4.8	33.3
6	20	5.3	5.2	33.1
7	50	5.7	5.6	33.2
Theory....				33.2

* In solutions more acid than pH 4.0 the results are low; 50 cc. of sodium acetate are not an excess and give correct results.

of calcium at varying acidities and determined the amount by both the gravimetric and volumetric methods. By this procedure it is possible to determine faulty results and also the cause of error. Titration with permanganate determines the oxalates. If phosphates are present and contaminating the calcium oxalate they will not affect the result. By the gravimetric method one determines the calcium as oxide and the oxalates are destroyed. Phosphates will cause the results to be too high. Reasoning thus, one can deduce the following:

Precipitate consisting of:	Method.	
	Gravimetric.	Volumetric.
Calcium oxalate alone..............................	Correct.	Correct.
" " and calcium phosphate............	High.	Low.
" " " magnesium ammonium phosphate.......................................	"	Correct.
Calcium oxalate and magnesium oxalate............	"	High.
" " " " hydroxide.........	"	Correct.

Therefore, we took samples of calcium chloride. To these we added phosphates and made determinations by both volumetric and gravimetric methods at pH 7.4. Correct results were obtained by both. To a second sample we added magnesium sul-

fate and made determinations by both methods at pH 7.4. Correct results were obtained by both. Then to a third series of samples we added both phosphates and magnesium. The acidity was adjusted by ammonia to pH 5.6 to 7.4. The results are given in Table III.

From these data it is clear that in the precipitation of calcium oxalate, neither magnesium oxalate nor hydroxide give false results if no phosphate is present. Calcium phosphate does not give false results even in slightly alkaline solution when no magnesium is present; but if magnesium is also present, magnesium ammonium phosphate precipitates if the solution is more alkaline than pH 5.6.

TABLE III.

*The Least Acid Limit for Precipitating Calcium Oxalate.**

Substance present.	pH	Gravimetric Ca.	Volumetric Ca.	Ca present.
		mg.	*mg.*	*mg.*
Ca and PO_4..................	7.4	84.9	84.4	
Ca and Mg..................	7.4	84.8	84.4	
Ca, Mg, and PO_4..............	5.6	84.9	84.7	
" " " "	6.0	86.0	84.5	
" " " "	6.6	92.0	84.9	
" " " "	7.0	94.3	84.9	
" " " "	7.4	100.2	84.8	84.6

* The least acid limit for the determination of calcium oxalate by the gravimetric method is pH 5.6. At points less acid magnesium ammonium phosphate is precipitated.

The Best Method of Obtaining the Desired Acidity.—When sodium acetate is added to a mixture of hydrochloric and oxalic acids, the stronger acids form salts with sodium and there remains in the solution acetic acid in the presence of acetates. This is an excellent buffer mixture and regulates the hydrogen ion concentration. If one knows the ratio of the acetic acid to the sodium acetate, with Walpole's (15) chart, one can estimate the pH. This is given in Table II under the column "pH calculated." How closely this approximates the pH as actually determined is shown by comparing these values with those given in the same table under the column "pH determined." As the ratio of acetic acid to sodium acetate becomes smaller the pH approaches 7.0 as a limit.

McCrudden's fear that calcium phosphate would precipitate unless there were between 6 and 10 cc. of sodium acetate is groundless. To test this point experimentally we made up a solution containing 2 cc. of 10 per cent calcium chloride, 2 cc. of 10 per cent magnesium sulfate, and 2 cc. of 10 per cent sodium acid phosphate. These we precipitated according to McCrudden's directions: 10 cc. of 0.5 N hydrochloric acid, 10 cc. of 25 per cent oxalic acid, 10 cc. of 3 per cent ammonium oxalate, cooled and to them added varying amounts of 20 per cent sodium acetate solution. The results are shown in Table II. Thus, even 50 cc. of acetate are not an excess and give correct results.

We found, by experiment, that it makes no difference whether one regulates the acidity by acetic acid and sodium acetate mixtures or more conveniently and quite as accurately by adding ammonia to the acid phosphates present. We have, therefore, developed a method based upon this conclusion, the details of which are given in the following paper.

Limits of Acidity.—From the above discussion it is clear that the limits of acidity for the precipitation of calcium by oxalic acid in the presence of magnesium and phosphates are determined. The most acid limit is pH 4.0. If the solution is more acid than this the calcium oxalate is partly converted into acid calcium oxalate which is so soluble that it is not quantitatively precipitated. The least acid limit is pH 5.6. If the solution is less acid appreciable amounts of magnesium ammonium phosphate precipitate and contaminate the calcium oxalate.

SUMMARY.

The hydrogen ion concentration is an important factor in making a calcium determination. If the solution is more acid than pH 4.0 calcium oxalate is dissolved. If the solution is less acid than pH 5.6, magnesium ammonium phosphate is precipitated.

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A RAPID AND ACCURATE METHOD FOR CALCIUM IN URINE.

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McCrudden's method (1, 2) for the determination of calcium in urine, at present probably the most accurate and satisfactory of all the calcium methods, is open to the serious criticism that it requires platinum dishes, and is laborious and time-consuming. Not infrequently it takes 24 hours to filter the urine and wash the precipitate. After filtration the ashing and weighing usually occupy an additional day, sometimes longer. McCrudden endeavored to shorten the method by titrating the precipitated calcium oxalate with potassium permanganate, but as the precipitate was contaminated with uric acid obtained too high readings. He states:[1]

> "I have concluded, therefore, if calcium oxalate, precipitated from urine, is contaminated with uric acid, as indicated by the reddish color of the precipitate, the calcium should not be determined by titration."

It is very questionable whether color is a good indication of the presence or amount of uric acid, since pure uric acid is colorless. The urinary uric acid, as is well known, depends for its coloration upon the presence of urinary pigments. Because of the above warning, and our experience, we have always relied on McCrudden's method only after ashing and weighing the calcium as calcium oxide. We felt that his method would be improved if we could avoid gravimetric analysis. Such a rapid clinical method for the determination of calcium in the urine is, therefore, desirable.

The method about to be presented, has the advantage of increasing the speed of the determination without appreciably

[1] McCrudden (2), p. 194.

decreasing the accuracy. The problem before us was to precipitate the calcium as calcium oxalate avoiding contamination with uric acid, and then to titrate the precipitate with potassium permanganate.

We first attempted to remove the uric acid by filtration after adding sufficient acid to convert the urates to uric acid and also to dissolve any calcium oxalate present. This is inadequate since considerable time is required and uric acid is very slowly and not quantitatively precipitated from urine. We also object to filtration, which McCrudden recommends for an initial procedure without adding acid, on the ground that any calcium combined with oxalic acid would be held back by the filter paper and lost.

We then searched for some solvent of uric acid with which the precipitate of calcium oxalate and uric acid might be washed on the filter or which would keep the uric acid in solution. Various solvents were tried. Hot sodium carbonate readily dissolved the uric acid but caused the readings to be much too low. Hexamethyleneamine was found to be very inefficient. Piperidine, a noted uric acid solvent, rendered the urine highly alkaline, dissolved the uric acid by virtue of its alkalinity, and was therefore inapplicable. No satisfactory solvent was found.

To eliminate the uric acid, the urine could be either dry ashed or ashed in the wet way following the Neumann method (3). Both these methods have been utilized in determining calcium. There is no objection to them except for the time and labor involved. We thought we might save time and avoid technical difficulties; and if we could destroy the uric acid by oxidation, we would accomplish in a short time, without evaporating the urine, the same purpose as ashing. Any substance which would reduce permanganate would be removed by oxidation.

We tried concentrated solutions of potassium permanganate on theoretical solutions containing calcium and uric acid with correct results. With urine, however, a surprising amount of this reagent was needed for complete oxidation. And this left large quantities of manganous ions, which, in the presence of oxalate ions, were difficult to hold in solution. It is not impossible that this method may be applicable, since manganous oxalate, like magnesium, is held in solution by ammonium chloride (4). We sometimes obtained an impure precipitate.

We then used ammonium persulfate and obtained excellent results (5). This reagent has the advantage of being relatively cheap, of being a strong uric acid-oxidizing agent, and an inefficient oxalate-oxidizing agent. In fact on theoretical grounds it is practically ideal for the purpose. When oxidation is complete, instead of manganous ions, we have sulfate and sulfite ions in solution, and these are not objectionable.

Ammonium persulfate is a white crystalline, deliquescent substance, which deteriorates on exposure to the air. When first put into solution it crackles with the evolution of a gas that smells like ozone. When added to the urine it froths. These properties we have taken as a rough index of its activity. A sample we obtained from Eimer and Amend was very satisfactory, but contained an appreciable amount of calcium. A blank should be run, therefore, for the calcium content. The reagent oxidizes best in an acid medium.

After the urine has been oxidized the calcium is precipitated as oxalate. The method as suggested by McCrudden may be followed.[2] He uses sodium acetate to regulate the acidity. We carry out the precipitation between definite limits of hydrogen ion concentration (6). We have found that the optimum lies between pH 4.8 to 5.2. Therefore, we use 1 drop of methyl red as an indicator. If the color fades rapidly we add a second drop of indicator solution. This indicator almost exactly covers the desired range of pH. It gives a red color when the acidity is as great or greater than pH 4.6, a yellow color when the acidity is less than pH 6.0, and an intermediate color between pH 4.8 to 5.4. This is the color desired. When one uses methyl red as an indicator one may use sodium acetate to decrease the acidity but it is not necessary. We use NH_4OH and bring the solution to the proper end-point, as shown by the color of the indicator. The important condition is to have the solution at the proper hydrogen ion concentration.

When the calcium has been precipitated as described above it is allowed to stand over night. (McCrudden shakes the mixture 10 minutes instead of allowing it to stand.) It is then filtered. Halverson and Schulz (7) have suggested filtering through a

[2] McCrudden (2), p. 199.

Gooch crucible made with specially prepared and ignited asbestos. This is unnecessary; for if the urine has been oxidized with persulfate it filters almost as readily as water. A good grade of hardened filter paper is easier to use. When several determinations are made at once it is practically as rapid as preparing the Gooch crucible and there is no chance of the filter's not retaining the precipitate. The precipitate is then washed back into the original flask and titrated with permanganate.

Reagents required.	
Ammonium persulfate.	Nitric acid (sp. gr. 1.42).
Oxalic acid, 2.5 per cent.	Sulfuric acid (sp. gr. 1.84).
Methyl red, 0.02 per cent in 50 per cent alcohol.	Ammonium hydroxide (sp. gr. 0.9).
Potassium permanganate, 0.05 N.	Sodium oxalate, Sörensen 0.05 N.

Details of the Method.

1. To 100 cc. of unfiltered urine in a 250 cc. Erlenmeyer flask add 5 cc. of concentrated HNO_3 or H_2SO_4, and one spoonful containing 3.0 to 4.0 gm. of ammonium persulfate. Insert a funnel in the flask to prevent spattering.
2. Boil and keep near the boiling point on a hot plate, or over a low flame, for 1 hour, or until reduction of the ammonium persulfate is complete, as evidenced by an absence of frothing when the flask is agitated. The solution at this point is pale green in color.
3. Add 10 cc. of 2.5 per cent oxalic acid.
4. Cool to room temperature.
5. Neutralize with ammonium hydroxide, using 1 drop of methyl red, as an indicator.
6. Cool to room temperature.
7. If the color is now red, the solution may be brought to the desired color by a few drops of ammonium hydroxide. pH 4.8 to 5.2.
8. Let stand over night (McCrudden shakes the mixture for 10 minutes instead of allowing it to stand).
9. Filter. Whatman No. 50 hardened filter paper, 12.5 cm., has been found satisfactory. Wash the precipitate and flask three times with distilled water, filling the filter two-thirds full each

time and allowing to drain. Break a hole in the filter paper, and wash back the precipitate into the original flask, first with distilled water, and then with hot dilute sulfuric acid, bringing the volume to about 100 cc.

10. Add 10 cc. of concentrated sulfuric acid, and heat to 70–80°C.

11. Titrate with 0.05 N potassium permanganate, taking as an end-point the first color that persists 15 to 30 seconds. If, as occasionally happens, the precipitate is colored red by the methyl red, and so colors the solution to be titrated with permanganate, this color does not interfere with the end-point, as it is quickly oxidized. The indicator is not present in sufficient amount to cause any appreciable difference in the titration. The usual precautions should be taken of standardizing the permanganate each day with standardized oxalate. 1 cc. of 0.05 N $KMnO_4$ = 0.001 gm. or 1 mg. of calcium.

EXPERIMENTAL RESULTS.

In the experiments here given no effort was made to obtain the highest degree of accuracy; they represent that obtainable in any physiological laboratory by an analyst of moderate experience. In any series of determinations reported no results have been omitted.

Our standard solutions of calcium were made from calcite, and checked by McCrudden's gravimetric method. A solution of $CaCl_2$, which contained 0.0830 gm. of Ca per 25 cc., was checked by McCrudden's method and gave the following results: 0.0831, 0.0832, 0.0831.

The results on theoretical solutions of calcium are given in Table I. A sample of urine, to which uric acid was added, analyzed by McCrudden's gravimetric, McCrudden's volumetric, and the authors' methods, yielded the results shown in Table II. On another sample of urine the calcium content was determined by the authors' method. To portions of it were added known amounts of calcium. The results are given in Table III. Six determinations were then made on a sample of urine by the authors' method and six determinations on the same urine by McCrudden's gravimetric method. One of these latter was unfortunately lost. The results are given in Table IV.

TABLE I.

Theoretical Solutions by Authors' Method.

Calcium taken.	Uric acid.	Ammonium persulfate.	Calcium present.	Calcium found.
gm.	*gm.*	*gm.*	*gm.*	*gm.*
0.0000	0.0	4.0	?	0.0002
0.0332	0.0	4.0	0.0334	0.0337 0.0332
0.0332	0.2	4.0	0.0334	0.0334 0.0339

TABLE II.

Urine Plus Uric Acid.

Urine.	Uric acid.	Method.	Calcium.
cc.	*gm.*		*gm.*
100	0.2	McCrudden's gravimetric.	0.0342 0.0345 0.0345
100	0.2	McCrudden's volumetric.	0.0541 0.0472
100	0.2	Authors'.	0.0344 0.0342 0.0346

TABLE III.

Recovery of Added Calcium by Authors' Method.

Urine.	Calcium added.	Calcium obtained.	Added calcium recovered.
cc.	*gm.*	*gm.*	*gm.*
100	0.0000	0.0113 0.01165 0.0116	
100	0.0165	0.0272 0.0284 0.0274	0.0162

TABLE IV.

Calcium in Urine.

Urine.	McCrudden's gravimetric method.	Authors' method.
cc.	*gm.*	*gm.*
100	0.0152	0.0160
	0.0168	0.0159
	0.0176	0.0161
	0.0158	0.0158
	0.0148	0.0161
	Lost.	0.0159
Average.............	0.01604	0.01596

DISCUSSION.

The method yields correct results with theoretical solutions containing calcium and uric acid. The calcium in urine can also be determined accurately and added calcium is recovered. Added uric acid does not affect the results by our method but gives high results if not oxidized. The advantages of the method described are: that by oxidation of the urine the resulting solution filters nearly as rapidly as water; the conditions for the precipitation are put on a rational basis by bringing the solution to the proper hydrogen ion concentration, instead of adjusting the acidity empirically with sodium acetate; the removal of the uric acid by oxidation permits the rapid and convenient method of titrating the calcium oxalate with potassium permanganate, instead of conversion to calcium oxide and weighing; and it permits rapid and accurate determinations of calcium in urine without requiring platinum. The method is accurate within 1 per cent in determining 30 mg. of calcium.

CONCLUSION.

1. Calcium in the urine can be accurately determined, if the urine is oxidized with ammonium persulfate.

2. The calcium is precipitated as the oxalate at pH 4.8 to 5.2, and titrated with 0.05 N potassium permanganate.

3. The method requires less than one quarter the time necessary for gravimetric determinations.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

SIXTEENTH ANNUAL MEETING.

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REASONS FOR BELIEVING THAT RESPIRATORY X IS NOT C_H.

By YANDELL HENDERSON.

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Low oxygen stimulates respiration. So also does CO_2, but through the C_H of the blood. Following the principle of economy of causes most investigators have held that low oxygen must also alter the C_H of the blood or of the cells of the respiratory center itself. Against this view is the fact that low oxygen induces not acidosis but alkalosis; also the fact that CO_2 stimulates respiration even after vagus section, but low oxygen apparently does not. Under morphine the responsiveness to CO_2 is greatly reduced, while low oxygen is still an effective stimulant.

Respiratory X is the hypothetical substance occurring in the blood under low oxygen. It is not an acid. Some evidence suggests that it does not act upon the respiratory center but stimulates the pulmonary vagal endings. It might be a sulfur compound, for H_2S is a powerful respiratory stimulant capable of causing overbreathing followed by fatal apnea. After vagus section sulfide is merely depressant; its stimulant action is, therefore, like that which respiratory X, by hypothesis, should have upon the pulmonary endings. Sulfide is rapidly oxidized in the blood. It does not act through C_H. Recent, and as yet, unpublished investigations, indicate that low oxygen must act through a substance of these physiological properties, although apparently respiratory X cannot be simply H_2S.

The following experiments were mentioned: On Pike's Peak merely squeezing a hand bulb or even opening and closing the fist sufficiently rapidly to fatigue the forearm caused, in Y. H. and some others, but not in all persons, marked hyperpnea. At sea level a similar experiment can be performed, but only on very sensitive subjects, by sawing wood, using both arms vigorously, but no other muscles. The significant fact is that the hyperpnea that is induced is followed by apnea. Apparently some substance, in addition to CO_2, is produced in the overworked and

thus in anoxyemic muscles, which passing into the blood causes overbreathing. It is quite certain that the condition induced in the blood by overbreathing is alkalosis (low ratio of H_2CO_3: $NaHCO_3$), not acidosis. Therefore, the substance is not an acid, but a respiratory stimulant of some other type.

CARBON DIOXIDE AS AN INHIBITANT OF CELL GROWTH.

By G. H. A. CLOWES and HOMER W. SMITH.

(From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.)

Comparative experiments carried out on dividing sea urchins' eggs indicate that carbon dioxide exerts a vastly greater inhibitory action than mineral acids, and that the effect of carbonic acid bears no relation to the hydrogen ion concentration. In a single series of experiments carried out under comparable conditions, carbon dioxide exerted an inhibitory effect at pH 8, and completely prevented cell division at pH 6.3, while hydrochloric acid exerted little or no inhibitory effect at pH 5.8 and development was still found at pH 4.6.

A similar difference is observed in studying the inhibitory effect of acids on the maturation of the starfish egg. It is suggested that this specific influence of carbon dioxide is attributable to its greater solubility in organo substances than in the aqueous phase and the experiments indicate that carbonic acid may play an extremely important rôle as a specific regulatory substance in cell growth.

THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

By F. S. HAMMETT and J. E. NOWREY, Jr.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

The subcutaneous injection of germanium dioxide solutions in adult male or female albino rats produces a marked erythrocythemia. There is no evidence of any toxic action of this compound, even when given in doses up to 180 mg. per kilo of body weight. The erythrocythemia persists for at least 5 weeks after the last dose when large doses are given. It persists for at least

2 weeks after the administration of relatively small doses. There is no accompanying leucemia.

Histological preparations of the liver, spleen, and bone marrow show the following effects have been produced. There is in most cases a dilatation of the hepatic capillaries and a relative engorgement of these with erythrocytes. There is no evidence of the taking on by the liver of its pristine erythropoietic function. The impression is given by the sections cut from the spleens that here too there is an increased number of erythrocytes which is accompanied by a more dense accumulation of cells in the Malpighian corpuscles. This, however, is merely an impression and not of significant magnitude to be validly distinctive. There is neither evidence for an increased red cell destruction by the splenic phagocytes nor evidence that new red cell formation is taking place in this organ. In the bone marrow there is ample evidence that this tissue has been stimulated to the formation of a larger number of nucleated erythrocytes than is taking place in the bone marrow of the control animals. This is verified by counts made of these types of cells.

An examination of the smears made for the determination of the differential counts of the white blood cells showed an entire absence of nucleated red cells. Nevertheless, it was found that the smears from those rats which had received the germanium injections contained more young red cells per unit area than did the smears of the controls. The index of the young red cells was the presence of polychromatic staining erythrocytes.

On the basis of these findings it is concluded that germanium dioxide is an erythropoietic agent of remarkable potency and that the source of the erythrocythemia produced by it is not a liberation of cells from some deposit within the organism, but is a formation of new cells by the bone marrow which has been stimulated to increased activity by the compound used.

IS THERE A SUBSTANCE OTHER THAN FAT-SOLUBLE A ASSOCIATED WITH CERTAIN FATS WHICH PLAYS AN IMPORTANT RÔLE IN BONE DEVELOPMENT?

BY E. V. McCOLLUM AND NINA SIMMONDS,

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AND P. G. SHIPLEY AND E. A. PARK.

(From the Departments of Pediatrics, the Johns Hopkins University, Baltimore, and School of Medicine, Yale University, New Haven.)

A series of experiments has been conducted which was designed to show the comparative values of butter fat and of cod liver oil in protecting young rats against the injurious effect of a pronounced deprivation of calcium. The basal diets contained about one-fifteenth the optimal amount of calcium and nearly the optimum of phosphorus.

Cod liver oil exerts a very marked protective influence, enabling the animals to grow, be fairly fertile, and appear well nourished for a considerable period under dietary conditions where without it they fail to grow at all and die early. We were unable to demonstrate much difference in the degree of protection afforded by 1 and 3 per cent of the oil.

With the same diets containing butter fat instead of cod liver oil very little protection was afforded even when 3, 10, and 20 per cent of fresh butter fat was supplied.

When in a series of experiments the calcium content of the diet was increased by small increments from 0.045 to 0.245 per cent through the addition of calcium carbonate, the differences between the nutritive effects of cod liver oil and of butter fat tend to disappear and do entirely disappear as the calcium content approaches the optimal, which is about 0.641 per cent.

The results suggest that either (*a*) cod liver oil contains in abundance a dietary essential which is contained but sparingly in butter fat, or (*b*) that at least two organic nutritive principles are associated with certain fats, and that both butter fat and cod liver oil contain each, but in proportions widely different.

THE EFFECT OF DIETS VERY HIGH IN PHOSPHORUS AND VERY LOW IN CALCIUM ON THE DEVELOPMENT OF THE BONES IN YOUNG RATS.

By E. A. PARK and P. G. SHIPLEY,

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and E. V. McCOLLUM and NINA SIMMONDS.

(From the Laboratory of the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

Rats fed diets low in calcium but high in phosphorus grow slowly and remain stunted. They seem nervous, reacting in an exaggerated manner to slight stimuli. One or two of them died suddenly when handled. The behavior taken in conjunction with the composition of the diet naturally suggested the possibility of tetany.

The bones were thin. The enlargements of the costochondral junctions and ends of the long bones of the limbs were slight or lacking. Fractures were not found.

Microscopically, the bones showed a fairly constant condition. The cartilage was in most instances calcified. The trabeculæ were thin and exceedingly numerous both at the epiphyseal end of the shaft and in the cortex. The trabeculæ were bordered with not very thick zones of osteoid. A thin fibrous tissue invested the trabeculæ, and when it filled in the spaces between the trabeculæ, it gave rise to pictures corresponding to the "fibrous marrow" so commonly seen in rickets. Scattered around the trabeculæ were many mononuclear cells with basophilic granules, evidently derived from the fixed tissues. They were not found in the marrow but only in the vicinity of the trabeculæ; in our experience they are seen in the rat when fed diets which are low in calcium. Everywhere in the trabeculæ were encountered evidences of bone destruction for the most part carried on by small perforating blood vessels resembling those which destroy the cartilage at the cartilage shaft border. Trabeculæ were found which were very well marked by holes or pits with irregular margins and often containing fragments of bone and bone corpuscles. Other trabeculæ have lost an entire side apparently by process of erosion. Evidently when the diet is deficient in calcium, the

organism keeps replenishing the calcium in the circulating blood. The histological changes in the bones mentioned were remarkably constant irrespective of the diets employed. The histological changes in the bones which have been mentioned seem, therefore, to have been dependent on the quantities and relations of the calcium and phosphorus present.

Different diets have different rickets-producing properties apart from their contents of calcium and phosphorus. For the production of rickets there must be growth, not growth in weight, but growth of the skeleton. We believe it probable that a certain amount of the fat-soluble organic factor in the diet favors the development of rickets and is essential for its development; it now seems probable that a certain amount of the fat-soluble factor in the diet is essential for the development of the rickets.

Certain diets low in calcium and having phosphorus in concentrations not far from the optimal, when fed to the rat under the experimental conditions already mentioned, produce rickets. When the phosphorus in the diet is increased to high concentrations, whether by the addition of inorganic or organic phosphorus, the pathological condition in the bones appears to recede from rickets. Diets low in calcium but high in phosphorus seem to be, therefore, less effective in the production of rickets than diets low in calcium in which the phosphorus concentration is not far removed from the optimal.

NEW METHOD FOR THE DETERMINATION OF URIC ACID AND EVIDENCE AS TO ITS DIMORPHISM.

By J. LUCIEN MORRIS and A. GARRARD MACLEOD.

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During critical investigation of colorimetric methods for the determination of uric acid certain conclusions were reached concerning the chemical principles underlying the reactions involved. The zinc precipitation of uric acid possesses many advantages over the silver precipitation. Sodium cyanide, in addition to its usefulness in forming double ions with silver or zinc, multiplies the amount of color obtainable from a given amount of uric acid. By use of this multiplying effect a definite maximum color is

attained when an excess of cyanide is present. When such a quantity of cyanide is used presence of sodium carbonate is undesirable. Cyanide gives a color with phosphotungstate in the absence of sodium carbonate. Among other conjugated tungstic acids, arsenotungstic acid was prepared and was found to obviate this difficulty. A new method, based upon these observations, was devised which possesses many advantages over former procedures.

Comparative results were secured from a large number of specimens of human blood by application of the new and the older methods. Some cases showed agreement but others showed marked irregularities. These irregularities were higher values obtained by the new method than by the old. All efforts to lower the new method results obtained in these cases failed. A means was found, however, by which the older method results could be increased so as to correspond with the new method results in those cases in which irregularities occurred, but the same means did not increase the values in the cases in which the two methods agreed. The nature of the modification of the old method used for this purpose suggested that the additional value was due to the presence of a second form of uric acid rather than any other substance. Further evidence to substantiate this conclusion is that uric acid was separated as such from large quantities of human blood in amount corresponding to the new method value.

A PHYSICOCHEMICAL METHOD OF CHARACTERIZING PROTEINS. III.

By EDWIN JOSEPH COHN.*

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In two earlier reports to this society we have pointed out: (1) that curves representing the fractional titration of proteins with acids and bases give a physicochemical method of characterizing proteins, and (2) that a comparison of the titration curves of a number of proteins showed differences in slope at their isoelectric points. This difference in slope was correlated with their solubility. It was shown that the slope of the titration curve of

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egg albumin (a protein that is soluble in water), was steeper at its isoelectric point than those of the slightly soluble globulins, and that the slope of glutenin (a protein insoluble in water but soluble in dilute acids and alkalies), was still less steep.

The titration of proteins, that are largely precipitated in the neighborhood of their isoelectric points, involves the heterogeneous equilibrium between precipitated and dissolved protein. This is being quantitatively studied in a number of cases by analysis of the concentration of total protein, of protein ions, and of hydrogen ions in the liquid phase.

It has been found:

1. that tuberin, euglobulin, pseudoglobulin, and casein have constant solubilities at their respective isoelectric points. The solubility of these proteins has been found to be independent of the amount of undissolved protein present, and approximately equal to:

	Per liter.
	gm.
Tuberin	0.4
Euglobulin	0.2
Pseudoglobulin	0.1
Casein	0.08

This solubility (S) must be considered to be made up of undissociated protein molecules, (HPOH), and of the dissociated protein ions, (PH^+) and (POH^-).

2. Upon the addition of an acid or an alkali to one of these proteins the solubility increases. In the case of casein it has been found that the increase in solubility is, within wide limits, proportional to the NaOH added. From this observation it follows that the amount of protein dissociated as base (HP^+) is negligible, and that the solubility of the undissociated molecules (HPOH), remains constant, as long as the heterogeneous equilibrium with the casein precipitate persists.

3. As long as the solubility of the undissociated protein is constant the mass law equation should assume the form:

$$(H^+) \times (BPOH) = K$$

where (BPOH) designates the soluble compound of protein and base, and K the solubility product constant. This equation has been found to hold for casein in the earliest stages of the reaction:

$$(\text{Casein}) + (\text{NaOH}) = (\text{Na caseinate})$$

where the protein may be considered as acting as though it were a monovalent acid. It varies, however, with variation in the protein concentration in a manner that is still being investigated.

The titration curve for casein has recently been determined by Loeb, and confirmed in this laboratory. This curve appears to have the hyperbolic form (characteristic of the solubility product) only for a short distance near the isoelectric point. Thereafter the curve assumes the S-shape characteristic of homogeneous buffer action. If the curve describing the solubility product is extrapolated, on the basis of the constant calculated in the early stages of the reaction, the amount of combination with base due to this group of valences may be subtracted isohydrically from the titration curve. It will then be found that a second curve is obtained still more characteristically S-shaped. By this graphical method casein may be seen to dissociate as an acid of at least two different strengths.

METABOLISM OF CALCIUM AND PHOSPHORIC ACID ON ISORACHITIC DIETS.

By J. F. McCLENDON.

(From the Department of Physiology, University of Minnesota, Minneapolis.)

In young white rats the skeleton contains 96 per cent of the Ca and 82 per cent of the P of the body. The P:Ca ratio is 0.5 in the skeleton and 0.6 in the body. Normal rats of from 22 to 45 gm. on a normal diet retained 10 to 18 mg. of Ca per rat per day and 7 to 9 mg. of P. The same rats at a weight of 76 to 110 gm. retained 20 to 37 mg. of Ca and 11 to 14 mg. of P. This diet contained 790 mg. of Ca and 570 mg. of P. When the P is reduced Sherman has shown that rickets develops. The following diets are divided into isorachitic groups. Group 1 produces definite rickets under the conditions of little exercise and light. Group 2 was doubtful because of disease of the animals. Group 3 causes

very slow growth of the bones and Group 4 more rapid growth. Rachitic rats suffer from respiratory insufficiency due to small size of chest due to soft ribs.

Group 1.

Diet.	Flour.	Casein.	Spinach.	NaCl	"Lime."	Yeast.	Wheat germ Ca per 100 gm.	P per 100 gm.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*
57	85	10	1	2	2		931	148
59	93	0	1	2	2	2	894	138
60	90	0	6	2	2		971	125
61	91	2	1	2	2	2	905	132

Group 2. Diet 58 = diet 57 + 100 mg. of cod liver oil per rat per day.

Group 3. Diets 57 to 61 + 0.2 per cent flour substituted by KH_2PO_4.

Diet.	Ca	P
62	962	222
64	900	171
65	970	173
66	923	181

Group 4. Diet 63 = diet 62 + 100 mg. of cod liver oil per rat per day.

Litter 12. Body weight 28 to 58 gm. 1 month old when placed on diets.

Rat No.	Sex.	Diet.	P balance P. D.	Ca balance P. D.
			mg.	*mg.*
I	♀	57	−1	+2 Group 1.
III	♂	59	−1	+1
IV	♀	60	−2	+1
V	♀	61	−1	+4
II	♀	58	−3 (Pneumonia).	+2 Group 2.
VI	♂	62	+2	+2 Group 3.
VIII	♀	64	+1	+5
IX	♂	65	+1	+4
X	♀	66	+1	+4
VII	♂	63	+5	+12 Group 4.

THERMAL EFFECTS ACCOMPANYING ALTERATION OF THE O_2 AND CO_2 CONTENT OF BLOOD.

By HOWARD W. HAGGARD.

(From the Department of Applied Physiology, Yale University, New Haven.)

Experiments to be reported demonstrate that the reaction of reduced whole blood with O_2 or CO is practically athermic. The combination of laked blood or hemoglobin solutions with either gas is exothermic. The amount of heat actually liberated is influenced by the state of aggregation of the hemoglobin. Results to be reported suggest the probability that there is an endothermic absorption which neutralizes the liberation of heat by the combination of hemoglobin with O_2 or CO. The shift of the equilibrium $HbO_2 + CO \leftrightarrows HbCO + O_2$ under variation of temperature depends upon the relative heating effects of the combination of the iron of hemoglobin with O_2 and CO. Absorption of CO_2 by the blood is attended with the evolution of heat, and the loss of CO_2 by the absorption of heat.

CHANGES IN THE COMPOSITION OF THE IRISH POTATO TUBER DURING GROWTH WITH PARTICULAR REFERENCE TO THE INFLUENCE OF COPPER SPRAYS.

By F. C. COOK.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Three early varieties of potatoes, Irish Cobbler, Early Rose, and Early Ohio, and one late variety, the Green Mountain, were grown in northern Maine during the 1921 season. Some of the plants were sprayed with copper sprays and others received no copper sprays. Analyses of the tubers made during the growing season showed a gradual increase of solids, starch, and nitrogenous constituents. The principal constituent of the tuber is starch, where the chief increase takes place during tuber growth. The increase of nitrogenous constituents was pronounced in the diamino and other base nitrogen group, although the soluble and the coagulable nitrogen groups increased.

The increase of solids, starch, and nitrogenous constituents was greater in the copper-sprayed than in the non-copper-sprayed

tubers. The copper-sprayed tubers gave a higher average percentage of ash than the non-copper-sprayed tubers. A decrease of reducing sugar, sucrose, insoluble nitrogen, and insoluble ash was observed as the tubers developed.

The copper sprays showed their favorable effect at the time the first analyses were made, when the tubers were just large enough for analyses, being about 1 inch in diameter. The three early varieties of tubers showed a higher percentage of sugars (reducing sugars plus sucrose) than the Green Mountain tubers, a late variety, at the time of the first and second analyses. It appears that a correlation may exist between the high sugar content in the early stages of tuber development and the rapid growth which the early varieties make.

The copper sprays not only usually give an increased yield of tubers but yield tubers with higher food value; *i.e.*, with more solids which means more starch as the potato is a starch-producing plant. The nitrogenous constituents of the tuber are also increased by the application of copper sprays. These changes were found in 1921 when no *Phytophthora infestans* was present in northern Maine. The influence of the barium-copper spray on the potato appears to be particularly favorable. Data are presented in detail showing for the first time the changes which take place in tubers during their development and the favorable influence of copper sprays on them.

PEPSIN AND TRYPSIN OF TISSUES.

By H. C. Bradley.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

The examination of tissues for pepsin and trypsin, as enzymes of the autolytic complex (Dernby) fails to show evidence of either one. Crucial experiments were carried on in which only one or the other of these enzymes could function as a catalyst of protein hydrolysis and in which they were compared to similar digests to which pepsin or trypsin was added. The results confirm the older findings and definitely disprove the presence of these enzymes.

HYDROGEN ION CONCENTRATION IN AUTOLYSIS.

By A. KOEHLER, E. SEVERINGHAUS, and H. C. BRADLEY.

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.*)

Measurement of the H ion concentration in autolyzing liver shows that at death an H ion concentration of about pH 7+ exists. Acidity develops for 24 hours; reaching a pH of about 6.4 or 6.5. For 10 days this H ion concentration shows a slight progressive diminution to about 6.7 or 6.8. Thereafter there is a very slow but definite increase of H ion concentration, which may at the end of 20 days reach 6.4 or 6.5 again.

If alkali is added to the digest, sufficient to practically stop autolysis (say pH 9.0) there is a rapid increase of H ion during the first 24 hours (to 7.8 in this case) and thereafter a slow progressive increase, so that in 20 days the reaction may approximate 7.0 very closely. The rapid increase of H ion is not accompanied by autolysis nor by sufficient CO_2 production during the first few days to account for the change in reaction. Oxygen absorption during this period is large, however, suggesting the formation of lactates or similar intermediate acid products which neutralize the alkalinity.

Where acid is added sufficient to produce the optimum autolysis (H ion about 4.5 to 5.5) there is much less striking change of reaction. The increased buffer effect of rapid cleavage to amino-acids does not greatly alter the pH. A digest starting at 5.3 reached 5.7 in 4 days and 6.0 in 20 days, showing a gradual tendency of the reaction to approach that of control.

THE RELATIVE VALUE OF THE SOURCES OF BASE FOR THE FORMATION OF SERUM BICARBONATE.

By EDWARD A. DOISY and EMILY P. EATON.

(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.*)

Defibrinated blood which had been equilibrated with 18, 40, and 75 mm. of CO_2 was centrifuged under oil. Serum chloride and bicarbonate were determined and corrections were applied for the change in volume of the corpuscles. The loss of chloride accounted for 65 to 75 per cent of the increase of bicarbonate.

Separated serum was equilibrated with the same tensions of CO_2 and the bicarbonate determined. Our data indicate that not more than 15 per cent of the total increase of bicarbonate of serum in contact with its corpuscles is due to buffers of the serum. Phosphate determinations on the serum permit us to calculate their value as carriers of carbon dioxide. The serum proteins (including amino- and other organic acids) are probably responsible for most of the base of the serum which may be used to combine with carbon dioxide.

The two sources of base studied do not add up to 100 per cent. We are inclined to ascribe the other 10 per cent to a transfer of other anions (sulfate, phosphate, etc.) from serum to corpuscles.

DETERMINATION OF THE BICARBONATE OF THE BLOOD AND PLASMA.

By DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Concerning the acid-base balance more complete information can be obtained by determining both the pH and the bicarbonate of the blood as drawn, than by determining only the CO_2 capacity.[1] The bicarbonate concentration may be determined in two ways:

1. By determining the total CO_2 content, and calculating the proportion of CO_2 present as bicarbonate from the plasma pH. The equation of Hasselbalch[2] may be written in the form

$$[BHCO_3] = [\text{total } CO_2] \frac{10^{pH - pK_1}}{10^{pH - pK_1} + 1}$$

which may be used for calculation of the $[BHCO_3]$. The values of pK_1 have been determined by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke to be 6.15 for whole blood and 6.10 for plasma.

2. By adding excess acid to plasma, removing the CO_2, and titrating back to the original pH. This method is a modification of Van Slyke, Stillman, and Cullen.[3] 1 cc. of plasma is acidified

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 153.

[2] Hasselbalch, K. A., *Biochem. Z.*, 1917, lxxviii, 112.

[3] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.

in a round flask with 5 cc. of 0.01 N HCl, and the CO_2 is removed by whirling the mixture about the flask. The solution is transferred to a test-tube of 20 mm. diameter, where it is titrated. For the transfer 0.9 per cent NaCl is used, and the NaOH and HCl are also made up in 0.9 per cent NaCl solution, to prevent cloudy globulin precipitation at the end-point. The titration is carried to the color of a control tube in which 1 cc. of plasma has been mixed under oil with 20 cc. of 0.9 per cent NaCl solution containing phenol red. The volume of the titrated solution is also brought to 21 cc. at the end of the titration by adding saline solution.

This determination may be conveniently combined with the colorimetric pH determination[4] since the plasma solution used for the latter may also be used as control for the end-point of the titration.

THE COLORIMETRIC DETERMINATION OF THE pH OF BLOOD PLASMA.

By GLENN E. CULLEN.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The method is based upon the determination of the total correction which, applied to a colorimetric reading at room temperature in a known dilution of plasma or serum, gives the actual pH at 38°C. This correction factor includes correction for protein effect, the dilution effect, and for the temperature effect.

It is most convenient to dilute the plasma in 0.9 per cent saline solution to about 20 volumes. Dilution up to 15 volumes is accompanied by continual increase in pH, but beyond 20 volumes the change is slight. The temperature change between 20–30° is −0.01 pH per degree increase. The correction factor at 20°, based on about 40 parallel determinations performed colorimetrically and electrometrically on both human and horse blood, is −0.18 pH, with an average deviation of ± 0.02 pH. The calculation of the reaction at 38° from the colorimetric determination is as follows:

[4] Cullen, G. E., *J. Biol. Chem.*, 1922, l, p. xvii.

$$pH_{38^\circ} = \text{colorimetric pH to} + 0.01\ t^\circ - 0.38$$

The determination is carried out as follows: Blood taken under mineral oil, either oxalated or not, is centrifuged in a stoppered tube. The plasma is removed and always kept under oil. Two 20 cc. portions of neutral 0.9 per cent NaCl are placed in clear glass 20 mm. tubes. 7 drops of a 0.04 per cent phenol red solution are added. The saline solution is then covered with oil. With a 1 cc. pipette, graduated to deliver between two marks, 1 cc. portions of plasma are transferred to the two saline tubes. The mixtures are stirred and the color is compared in a comparator block with that of the Sörensen M/15 phosphate standards containing phenol red. These standards are made up at 0.05 pH intervals and the plasma color is read to 0.01 pH. The determinations are accurate to within the combined possible error of the colorimetric and electrometric determinations; *i.e.*, (0.02 + 0.02) = 0.04 pH.

This method may be applied to other protein-CO_2-containing solutions, such as joint fluids, etc.; but in such cases the protein correction must be redetermined.

A METHOD FOR THE DETERMINATION OF THE TITRATABLE ALKALINITY OF BLOOD.

By ISIDOR GREENWALD.

(From the Harriman Laboratory, Roosevelt Hospital, New York.)

The blood is laked, the proteins are precipitated with picric acid, and the mixture is diluted to ten times the volume of blood taken. An aliquot of the filtrate is titrated with 0.01 N NaOH, using methyl red, and neutral red, phenol red, or cresol red, and, finally, thymolphthalein. The total picric acid is precipitated with nitron, filtered, and weighed. The weight is converted into its equivalent volume of 0.01 N NaOH by multiplying by factors obtained by the titration of known amounts of picric acid. The titration of the free acid in the sample is subtracted from this. The difference represents the alkali contributed by the blood to neutralize the picric acid to the reaction shown by the indicator. The values obtained are unaffected by the presence of small amounts of oxalate, by the exact amount of picric acid used, by the degree of saturation of the blood with oxygen or carbon dioxide

and do not change rapidly as the blood is allowed to stand. The error is less than $\pm$ 2 per cent if the equivalent of 3 cc. of blood be used and is not more than $\pm$ 4 per cent if only the equivalent of 1 cc. be used. Normal blood yields the equivalent of from 40 to 47 cc. of N alkali per liter to methyl red and from 29 to 34 cc. to thymolphthalein.

A METHOD FOR THE INVESTIGATION OF TOTAL BASE EXCRETION.

By CYRUS H. FISKE.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

A method, sufficiently rapid and calling for small enough amounts of urine to meet the requirements of short period metabolism experiments, has been devised for the determination of the sum of all the non-volatile strong bases in urine (sodium, potassium, calcium, and magnesium). It is based partly on well known and long used devices for the separation of these bases from other constituents (ashing with sulfuric and nitric acids, removal of phosphate with ferric chloride, and of the excess iron as the basic acetate) with certain refinements necessitated by the small scale of the method. The bases are eventually obtained as sulfates, and the determination is made by a sulfate estimation with the benzidine method.

PHYTIN AS A SOURCE OF PHOSPHORUS IN THE PREVENTION OF RICKETS.

By WALTER H. EDDY, H. R. MULLER, and HATTIE L. HEFT.

(From the Department of Physiological Chemistry, Teachers College, Columbia University, and the Department of Pathology, New York Hospital, New York.)

The announcement by Sherman and Pappenheimer of two diets (84 and 85) which are respectively rachitic and antirachitic has suggested the desirability of determining whether the antirachitic effect obtained by the addition of 75 mg. of phosphorus in 100 gm. of diet in the form of K_2HPO_4 is paralleled by other forms of dietary phosphorus. Phosphorus occurs in foodstuffs as inorganic phosphorus, as phosphoprotein, as phospholipin, and as

phytin. In papers soon to be published Pappenheimer, Zucker, McCann, and Gutman will report findings in regard to phosphoprotein and lecithin. The present paper deals with preliminary work on the rôle of *phytin* in this connection.

Microscopic sections are shown obtained from studies of a single litter of rats which indicate that when an amount of phytin is fed that furnishes about 75 mg. of phosphorus in 100 gm. of the basal diet, *i.e.* Diet 85 with phytin substituted in place of K_2HPO_4, protection fails. Growth facts and food intake figures are supplied that show that there was little difference in the growth rate of the three series studied and that the food intakes during the 30 day period were practically identical, 9+ gm. daily.

Preliminary results are reported on a much larger series of rats which at the time of preparation of this paper had been distributed over five series of diets as follows: Group I on Diet 84; Group II on Diet 85; Groups III, IV, and V on a diet which was modified by replacing the inorganic phosphate of Diet 85 by varying amounts of phytin, so proportioned as to provide 70, 105, and 140 mg. of phosphorus to each 100 gm. of the diet, respectively.

Figures were given for the inorganic blood phosphorus, as determined by the Bell and Doisy method, after 14 days on this diet. These figures seem to support the conclusions of Hess and Gutman in regard to the diagnostic value of this test as an indicator of rachitic conditions, if we consider solely the two control series. In the case of the phytin-fed rats, however, the figures obtained were extremely variable and failed to support the x-ray evidence. The x-ray evidence while confirming the efficiency and inefficiency of Diets 85 and 84 respectively, shows absence of protection in any of the phytin series. These results seem to suggest that even though we give phytin in amounts sufficient to double the amount of phosphorus supplied by the inorganic phosphate of control Diet 85 it fails to protect.

Previous experience has shown that final judgment should be reserved until microscopic sections are obtained and the above findings are reported as subject to revision in the light of such evidence at the end of the normal period.

THE INFLUENCE OF DIET UPON THE CONCENTRATION OF PHOSPHORUS AND CALCIUM IN THE SERUM OF RATS.

By BENJAMIN KRAMER and JOHN HOWLAND.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

A study of the calcium and the inorganic phosphorus in the blood serum of rats has been made to determine how the concentration of these substances is affected by diets, especially those producing rickets. We have previously shown that in human rickets the concentration of inorganic phosphorus is low and that it increases during spontaneous cure or as the result of the administration of cod liver oil. The concentration of calcium is essentially unchanged. In rats upon a diet deficient in phosphorus and in the organic factor contained in fats, especially cod liver oil (a diet which regularly produces rickets in the rat) the concentration of inorganic phosphorus is much diminished. The calcium is unchanged as long as a sufficient quantity of this is contained in the diet. When the diet is deficient both in calcium and in the organic factor, the calcium concentration of the serum is lowered. Whether there is a phosphorus or a calcium deficiency of the diet with a lowered concentration of these substances in the serum, cod liver oil administration assists greatly in maintaining the normal concentration of these elements.

THE FATE OF CERTAIN SULFUR COMPOUNDS IN THE ANIMAL ORGANISM.

By CARL L. A. SCHMIDT and GUY W. CLARK.

(From the Department of Biochemistry and Pharmacology, University of California, Berkeley.)

Experiments were carried out to determine the fate of certain sulfonic acid compounds, with especial reference to those concerned in bile metabolism. All substances were administered by mouth to dogs kept on constant diets. Taurine is excreted in the urine unchanged and not as taurocarbamic acid as claimed by Salkowski. Cysteic acid is deaminized but the remainder of the molecule is excreted in the urine unchanged. Administration of taurocholic acid does not lead to its appearance in the urine.

Isethionic acid is excreted in the urine, no oxidation of the sulfur to sulfate taking place. These experiments indicate that dogs can neither oxidize the sulfur in sulfonic acids to sulfates nor deaminize compounds in which the amino group is in the alpha position with respect to the sulfonic acid radical.

A NEW WEDGE COLORIMETER FOR THE COMPARISON OF SOLUTIONS CONTAINING TWO COLORS, AS IN THE COLORIMETRIC pH DETERMINATION.

A DEMONSTRATION.

By VICTOR C. MYERS.

(*From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.*)

It has previously been pointed out[5] that with the use of two wedges in a modified Hellige colorimeter, it is possible to obtain all the shades of color in a given indicator from the acid to the alkaline side, when one wedge is filled with an acid solution of the dye and the other with an alkaline solution, both being made with buffer solutions of a definite pH. The utilization of the wedges of the Hellige colorimeter for this work has been under consideration for some time in this laboratory.

Barnett and Barnett[6] and Gillespie[7] have utilized essentially the same principle in the colorimetric measurement of the hydrogen ion concentration. The former authors employ a long narrow rectangular glass box having a diagonal glass partition, one being used for the acid and the other for the alkaline solution of the indicator, while the latter achieves the same result by having a small movable cup fitted over the plunger but inside the cup of a Duboscq type colorimeter.

The use of wedges which are individually movable provide a much more flexible system. The reading of the wedge containing the dominant color of the dye, *e.g.* the red in phenol red, characterizes the hydrogen ion concentration, the yellow wedge being employed simply to obtain a correct color match. This

[5] Myers, V. C., *Proc. Soc. Exp. Biol. and Chem.*, 1921–22, xix, 78.

[6] Barnett, G. D., and Barnett, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 127.

[7] Gillespie, L. J., *J. Bact.*, 1921, vi, 399.

being the case it may also be employed to correct for any slight error due to extraneous yellow pigment in the unknown.

Briefly, the colorimeter comprises a brass box 30 cm. in height, containing a rack and pinion arrangement for three wedges, the movement of the wedges being entirely within the closed box. Readings are taken from 100 mm. scales which emerge from the top of the instrument as the wedges are raised. The instrument is provided with prisms and an eye-piece in front and a milk glass plate in back for the entrance of light. For the latter a small lamp box may be substituted. A door at the side gives access to the wedges and to the cup for the unknown which is mounted on it.

With one wedge the instrument may be used as an ordinary colorimeter. The second wedge provides for bicolorimetric work, as in the pH determination. To obtain a perfect match of unknown solutions which are slightly turbid or colored a third wedge may be used.

A PRELIMINARY REPORT ON THE NEPHROPATHIC ACTION OF THE DICARBOXYLIC ACIDS AND THEIR DERIVATIVES.

By WILLIAM C. ROSE.

(*From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston.*)

Comparative studies have been made of the influence upon renal function of a number of dicarboxylic acids administered subcutaneously as their sodium salts. Rabbits were used as the experimental animals. After a single dose of 1.0 to 1.5 gm. of tartrate, the preformed creatinine may increase six- or eightfold, and the blood sugar and cholesterol double in quantity, in addition to enormous increases in non-protein and urea nitrogen as previously observed by others. On the contrary, blood chlorides do not increase, and usually manifest distinct decreases in amount. Since tartrate nephritis is generally believed to involve the renal tubules primarily, the behavior of the chlorides may afford additional evidence for their elimination by the glomeruli.

In contrast to the action of sodium tartrate, sodium malate is only slightly nephropathic, and the salts of the closely related succinic and malonic acids are entirely without influence upon

renal function. On the other hand, sodium glutarate (1 to 4 gm.) produces a marked retention of nitrogenous blood ingredients, accompanied by a decided fall in phenolsulfonephthalein elimination. The nephropathic action of sodium glutarate explains the observation of Baer and Blum that following its subcutaneous administration to phlorhizinized dogs, decreases occur in the excretion of urinary nitrogen, sugar, and acetone bodies.

The toxic action of sodium glutarate cannot be due to a precipitation of the calcium derivative in the renal tubules, as has been suggested in explanation of the effect of sodium tartrate, since calcium glutarate is quite soluble.

We are extending our observations to other dicarboxylic acids.

THE RELATION OF SPLENECTOMY TO GROWTH AND APPETITE IN THE RAT.

By ARTHUR H. SMITH and LEAH ASCHAM.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

An experimental study of the alleged effect of splenectomy on the growth and appetite in the white rat was made, using the standard feeding technique in vogue in this laboratory. Observations on seventeen rats, eight of which were the progeny of splenectomized parents, gave no evidence of an increase in appetite or a variation from the normal rate of growth.

A study of the erythrocyte count on five splenectomized rats which were the progeny of splenectomized parents showed no anemia following the removal of the spleen.

CREATINURIA AND GROWTH IN THE DOG.

By VICTOR JOHN HARDING and OLIVER H. GAEBLER.

(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)

In studying the effect of diets varying composition upon the creatine excretion of young dogs, the authors have paid particular attention to the concomitant effect upon growth. Growth has been judged by alterations in body weight and in nitrogen balance. The change from a high protein diet to a low protein

diet had previously been shown to be followed usually by a decrease in creatine excretion, though if the low protein diet were continued long enough a rise in creatine ensued. The change from a low protein diet to a high protein diet, however, is not invariably followed by a rise in creatine excretion. The creatine excretion may rise, remain constant, or fall, depending very greatly upon the growth change induced by the alteration in diet.

Changes in growth, following the introduction of an essential amino-acid into a diet in which it was probably the growth-limiting factor, are accompanied by alterations in creatine excretion. Similarly, the introduction of vitamines into a diet, in which they had previously been deficient, by causing rapid growth changes will bring about changes in creatine excretion.

THE INFLUENCE OF FASTING AND OF LOSS IN BODY WEIGHT ON THE CARBOHYDRATE TOLERANCE.

By A. I. Ringer.

(From the Montefiore Home and Hospital, New York.)

In this investigation, the carbohydrate tolerance of diabetic patients was studied. The plan was to determine the tolerance when the patient presented himself and to repeat these tests at intervals of years, so as to find out in what way the progression of the disease and the loss in body weight might affect it.

The patient's blood sugar was determined in the morning before breakfast. After that he was given 50 gm. of bread with one cup of weak tea. The blood sugar was again determined after the first, second, and third hours. The urine was also examined for the presence of glucose.

The same test was repeated after 1 day's fast. Invariably after 1 day's fast, the patient showed ability to utilize carbohydrate better than on the day before the fast.

These same tests were repeated on four patients who presented themselves after having undergone courses of treatment in which their body weights were reduced from 15 to 22 per cent and during which period they were kept on low caloric diets and in an aglucosuric state.

After such examinations it was found that the patients that had lost in body weight not only had no increase in their tolerance for carbohydrates, but had a decided diminution in their power to utilize carbohydrates.

AN ISOLATION OF PHENOL FROM BEEF BLOOD AND FROM HUMAN BLOOD.

By ALICE ROHDE DAVIS and ELEANOR B. NEWTON.

(*From the Psychopathic Hospital, Boston.*)

Phenols have been isolated from normal urine and from blood in cases of phenol injection or phenol poisoning. We have found it possible to obtain phenol as a crystalline brominated compound from both normal human blood and beef blood using mercuric acetate and sodium acetate to obtain the difficultly soluble mercurated phenols. To a protein-free blood filtrate obtained by the use of heat and acetic acid followed by colloidal iron is added mercuric acetate in sufficient amount to make a 0.25 per cent solution and subsequently sodium acetate to make a 10 per cent solution. Phenol is partially precipitated together with mercury compounds of uric acid and of combined uric acid. On concentration a further yield of mercurated phenol occurs. These compounds may be decomposed with hydrogen sulfide in acid solution or by boiling potassium iodide solution. The free phenol is then distilled from acid solution. The distillate may have the odor of phenol and gives positive tests with ferric chloride, with Millon's reagent, and with bromine water. The precipitate of brominated phenol is recrystallized from alcohol and comes down in long needles which are colorless in small amounts and melt at 92° (uncorrected). The M. P. of tribromphenol is given as 91.5°.

ANTIKETOGENESIS.

THE KETOGENIC-ANTIKETOGENIC BALANCE IN MAN AND ITS SIGNIFICANCE IN DIABETES.

By P. A. SHAFFER.

(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis.*)

A revision of the values[8] assigned to the ketogenic versus antiketogenic influence of protein, carbohydrate, and fat in the metabolic mixture, allows the approximate calculation of the

[8] Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 457.

amount of total hydroxybutyric acid produced by the catabolism of any given mixture of foodstuffs. (The main change in the values is made necessary by the fact that each molecule of glucose is ketolytic for *two* molecules of acetoacetic acid.)

The fact that the calculated expectation now approximately agrees with the actual excretion of acetone bodies in nearly all of about a dozen different subjects so far examined—normals, moderate and very severe diabetics—indicates that the underlying hypothesis is substantially correct, and that the evaluations of ketogenic versus antiketogenic influences are not greatly in error.

From these facts it follows that the *minimum* amount of food carbohydrate needed to provide a theoretical ketogenic balance can be approximately calculated for any given subject; and this figure is the absolute minimum of carbohydrate tolerance below which ketosis is *unavoidable*. The most useful formula expressing the relationship appears to be:

$$\frac{\left(\begin{array}{c}\text{Total calories of energy exchange}\\ \text{per 24 hours}\end{array}\right) - (100 \times \text{urine N})}{50} = \begin{array}{l}\text{Gm. food CH to provide}\\ \text{approximate ketogenic}\\ \text{balance.}\end{array}$$

If a diabetic has enough carbohydrate tolerance to remain "sugar-free" on a diet containing the calculated amount (or more) of food carbohydrate he will have little or no ketosis. The calculated amount allows no margin of safety against accidental increase in total metabolism from unusual activity or infection.

If the tolerance is lower the *only* recourse is to reduce the total metabolism (the amount of ketogenic material in the metabolic mixture) by rest, low protein diet, undernutrition, or fasting, until the (approximate) relationship expressed above is accomplished. For each gram of the calculated minimum of carbohydrate which is not burned (because of low tolerance or because not available in food) slightly more than a gram of total hydroxybutyric acid is excreted.

A COMPARISON OF ACETONURIA CAUSED BY DISEASE WITH THAT CAUSED BY DIETS LOW IN CARBOHYDRATE.

By ROGER S. HUBBARD, SAMUEL T. NICHOLSON, Jr., and FLOYD R. WRIGHT.

(*From The Clifton Springs Sanitarium, Clifton Springs, N. Y.*)

Mathematical ratios were proposed to express the molecular relationship between glucose and fat for any diet fed. For normal cases receiving enough food to maintain metabolic equilibrium the ratio was based on the foods ingested; for diabetics not in metabolic equilibrium the ratio was based on the foods burned in the body, as calculated from the basal metabolism, carbohydrate ingested, and sugar and nitrogen excreted. Charts were shown in which the values of these ratios were compared with the acetone excretion, and a similarity of response was shown in the two conditions. From a study of the values of the ratio which corresponded with a very slight increase of the acetone excretion above normal, it was concluded that the glycerol radical of the fats probably figures as a source of antiketogenic material to the extent to which it will yield glucose when fed.

STUDIES OF THE METABOLISM OF DIABETES.

By RUSSELL M. WILDER, WALTER M. BOOTHBY, and CAROL BEELER.

(*From the Mayo Clinic and Foundation, University of Minnesota, Rochester.*)

A chart is presented giving the more important data of feeding experiments in a case of diabetes of great severity. These experiments were designed to throw light on the rôle of ingested protein on the diabetic metabolism. Observations were continued for 11 weeks with daily determinations of the urinary excretion of nitrogen, sugar, ammonia, acetone bodies, total acids, and phosphates and frequent determinations of the carbon dioxide-combining power of the plasma, the sugar, acetone, and fat content of the blood and the respiratory metabolism.

The following conclusions are submitted:

1. The postabsorptive or *basal* metabolic level of the diabetic individual is materially affected by the previous diet. In the

undernourished patient it may be found as low as 32 per cent below the Du Bois standard normal for a healthy person of like age, sex, and surface. The ingestion of food containing 1 gm. of protein per kilo of body weight with fat and carbohydrate in such an amount that the daily maintenance energy requirements of the patient were exceeded, caused an elevation of the basal level. The ingestion of 3 gm. of protein per kilo of body weight per day caused a greater rise in the basal metabolic rate than occurred with isocaloric amounts of other foods. A cumulation of the specific dynamic action of protein seemed to account in the main for the elevation of the *basal* metabolic level which occurred.

2. The rate of sugar utilization of this diabetic individual was depressed by high calorie diets, being much more markedly depressed by protein than by isocaloric amounts of fat. This protein effect was not primarily due to the sugar and ketogenic substances, which the ingestion of protein throws upon the metabolism, but to some other more specific action of protein.

3. Throughout this series of experiments the rate of sugar utilization varied inversely with the basal metabolic level of the patient, rising as the basal metabolic rate fell, and *vice versa*. This behavior suggests a definite interrelationship of the two and the fact that ingested protein elevates the *basal* metabolic rate makes it seem possible that it is by this mechanism that it adversely affects the rate of sugar utilization.

4. Diets relatively high in fat but low in protein, and planned to contain nearly 2 gm. molecules of fatty acid to 1 of glucose on two occasions checked and controlled a dangerous and rising acidosis. The data of the metabolism of 2 days when acidosis was minimal indicated that on these days the actual ketogenic-antiketogenic ratio in the metabolizing mixture was 1.53 and 1.78 respectively. It is suggested that the proportion of fatty acid which will completely burn with a limited amount of metabolizing glucose is not the same at all basal metabolic levels, but may be increased by measures designed to depress the basal metabolic rate.

THE EFFECT OF LOSS OF CARBON DIOXIDE ON THE HYDROGEN ION CONCENTRATION OF URINE.

By E. K. MARSHALL, Jr.

(From the Department of Physiology, the Johns Hopkins University, Baltimore.)

In certain conditions the escape of carbon dioxide from urine may have a marked effect in lowering the hydrogen ion concentration. Concentrated acid urines as ordinarily obtained on a mixed diet do not change appreciably in hydrogen ion concentration when shaken with air although practically all of the carbon dioxide may be given off. When very dilute urines or neutral and alkaline ones obtained after administration of alkali are so treated, there is a distinct decrease in acidity or increase in alkalinity. Precautions similar to those used in the case of blood are necessary in determining the pH of these urines. Experiments on man and animals indicate that the lowest hydrogen ion concentrations reported for urine may be too low due to neglect of proper precautions to avoid the loss of carbon dioxide.

A STUDY OF THE METABOLISM AND THE RESPIRATORY EXCHANGE IN POULTRY DURING VITAMINE STARVATION.

By R. J. ANDERSON and W. L. KULP.

(From the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.)

In an endeavor to obtain some information regarding the changes which occur in the metabolism of an animal which is deprived of the water-soluble vitamine B, a series of experiments was made on poultry. This study included observations under normal conditions of diet and then a state of vitamine starvation was induced by feeding polished rice until polyneuritis was finally produced.

A diet of polished rice caused a loss of appetite in our experimental animals and the food consumption fell to a low level. The continued lack of vitamine B in the diet caused a serious impairment of the digestive functions which during polyneuritis resulted in a practical cessation of digestion and assimilation.

During vitamine starvation there was a decided fall in the intensity of the metabolism corresponding to the decreased food consumption. The basal heat production in some cases fell to more than 50 per cent under that of the normal basal metabolism and the respiratory quotients averaged from 0.73 to 0.80 about 18 hours after feeding. Respiratory quotients approaching unity were obtained, however, during the first 3 or 4 hours after feeding polished rice until a short time before active symptoms of polyneuritis developed.

But during polyneuritis, although the crop of the fowl contained much undigested rice, the respiratory quotient seldom rose above 0.75, indicating a nearly complete inability at that time to utilize this food.

HEAT ELIMINATION AND GASEOUS EXCHANGE IN GRAPEFRUIT DURING STORAGE.

By C. F. LANGWORTHY and H. G. BAROTT.

(From the Office of Home Economics, States Relations Service, United States Department of Agriculture, Washington.)

The experiments here described belong to the same general series as those reported in 1919[9] for bananas and apples and in 1920 for celery and eggs, and like the earlier ones were carried out in the large respiration calorimeter of the Office of Home Economics. In the present case the tests, which were conducted December 2 to 10, 1920, were made with grapefruit such as are imported in large quantities from Porto Rico. Refrigerator ships cannot be obtained for this trade, the fruit is transported in the holds of ordinary vessels where there is no regular ventilation and the temperature averages from 75 to 80°F., and the loss through decay, molds, and similar causes is very great. The experiments were made at the request of and in cooperation with the Porto Rico Experiment Station in the hope that accurate observations of the heat elimination and gaseous exchange of the fruit under conditions comparable to those on shipboard might yield information of practical service to shippers.

123 kilos of commercial pack grapefruit were used. Single layers of these were placed in the calorimeter on shelves made of

[9] Langworthy, C. F., Milner, R. D., and Barott, H. G., *J. Biol. Chem.*, 1920, xli, p. lxix.

$\frac{1}{4}$ inch galvanized mesh wire and set 1 foot apart. Differential thermocouples were distributed among the fruit so that its temperature could be determined relative to its surroundings and resistance thermometers made it possible to determine the temperature of both the air within the calorimeter and of the walls. When the fruit had been placed in the chamber, the latter was sealed, the circulation of gases started, and the temperature adjusted to 78°F., at which point it was kept throughout the 8 days of the experiment. Determinations of the gaseous exchange and heat elimination were made at approximately 24 hour intervals, each determination representing the mean value for the period just ended.

The heat eliminated varied from 1.7 to 2.6 calories per hour for the total weight of fruit. The carbon dioxide elimination varied from 1.8 to 2.4 gm. per hour. The amount of water vapor given off was also very uniform, varying from 23.8 to 25.6 gm. per hour. The oxygen absorbed showed more variation and ranged from 0.7 to 3.1 gm. per hour. This irregularity in the oxygen may be accounted for by the fact that the oxygen is determined by difference and not measured direct as is the carbon dioxide and that with such small quantities of oxygen, a slight error in the residual analysis due to the large amount of moisture eliminated might appear as a large percentage error in the oxygen figure.

Since the heat elimination even under these experimental conditions with excellent ventilation and constant removal of heat, was sufficient to keep the temperature of the fruit above that of the surrounding air, it seems reasonable to suppose that where there is no ventilation, as in the hold of many ships carrying grapefruit, the temperature of the fruit would ultimately become several degrees higher than when it was placed on board.

A METHOD FOR THE DETERMINATION OF LACTIC ACID IN BLOOD.

By E. L. SCOTT and F. B. FLINN.

(From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York.)

The method which we describe is based upon that described by Ryffel. We have so modified the method that the filtrate obtained in Folin and Wu's system of blood analysis is used.

We have obtained satisfactory results in using the filtrate from 5 to 20 cc. of blood.

An aliquot of the filtrate is evaporated to about 40 cc. in an evaporating dish upon the water bath or if less than this, it is placed directly in a 500 cc. distilling flask with side neck and enough water added to bring it up to 40 cc. It is then neutralized with solid Na_2CO_3 and 45 cc. of concentrated sulfuric acid are added drop by drop, the flask being held under running water to keep it from heating. The flask is then connected with a Liebig condenser and a steam generator and distilled with a current of steam for 30 minutes, or until 150 cc. of distillate have passed over. The receiver is immersed in ice water. In this distillation the temperature of the contents of the flask is raised rapidly to 155°C. and kept between 155 and 158°C. during the entire process.

The distillate is then neutralized with 2 per cent NaOH solution and redistilled into an Erlenmeyer flask which contains 20 cc. of a 6 per cent solution of KOH and 20 cc. of 0.04 N standard iodine solution. This flask is also immersed in ice water. When 100 cc. have distilled over, the flask is removed and slowly warmed to about 20°C. 20 cc. of 15 per cent HCl are then added and the contents titrated with 0.04 N standard thiosulfate solution.

If care is taken to prevent heating during the addition of the sulfuric acid and to keep the temperature during the first distillation within the range indicated, the results are very concordant.

The thiosulfate solution should be standardized by treating a known amount of lactic acid in exactly the same manner as that described for the Folin and Wu filtrate.

THE BLOOD SUGAR CONTENT OF CAPILLARY BLOOD AS COMPARED WITH THAT OF VENOUS BLOOD.

By ISAAC NEUWIRTH AND ISRAEL S. KLEINER.

(From the Department of Physiological Chemistry of the New York Homeopathic Medical College and Flower Hospital, New York.)

Twenty normal students were employed as subjects. Blood was obtained simultaneously from a finger and from a vein of the same arm and was analyzed by the micro method of one of the authors (K). In eighteen out of the twenty cases the results did

not differ by more than 0.01 per cent and in the other two by only 0.02 per cent. The average of all twenty is 0.136 per cent for the capillary and 0.130 per cent for the venous blood. The method was also compared with a macro method and the conclusion is reached that the analysis of capillary blood by this micro method gives results which, for clinical purposes, can be considered identical with the venous blood sugar.

THE CALCIUM AND MAGNESIUM CONTENT OF THE HUMAN FETUS.

By MAURICE H. GIVENS and ICIE G. MACY.

(From the Research Laboratories, Western Pennsylvania Hospital, Pittsburgh.)

Twenty-five fetuses, ranging from 90 to 400 mm. in length and consequently varying from 3 to 8 lunar months of age, have been dried and ashed. The ash has been analyzed for its content of calcium and magnesium.

The increase in length of the body appears to be at a gradual rate at all times. The total solids of the body increase gradually and slowly up to the fifth month, thereafter at a more rapid rate; likewise is this true of the ash and its calcium content. The magnesium figures do not become significant until about the seventh month.

Up to the third month the calcium requirement of the fetus approximates *in toto* 300 mg. of CaO and 60 mg. of MgO. From this time up to the eighth month, the demand for lime is much greater, relatively and absolutely. The magnesium content of the body varies considerably. At no time does it equal or even approximate the amount of calcium present in the body.

The total ash varies from 3.85 to 33.4 per cent of the dry body weight. Calcium oxide represents from 1 to 12 per cent of the dry body weight and from 24 to 50 per cent of the total ash. In the majority of cases MgO is less than 1 per cent of the dry body weight and from 3 to 15 per cent of the total ash.

For the first 3 or 4 months of pregnancy the fetal demand for CaO varies from 10 to 30 mg. per day, whereas for the total period of pregnancy it may average 100 mg. per day. Whether or not the total lime requirement of the fetus can be met from the mother's food intake remains to be investigated.

THE EFFECT OF CHANGES IN THE PROTEIN AND ENERGY OF THE DIET OF MILKING COWS UPON THE MILK YIELD AND UPON THE AMINO N OF THE BLOOD.

By C. A. Cary.

(From the Research Laboratories of the Dairy Division of the United States Department of Agriculture, Beltsville.)

In five experiments on cows the changes in the blood by which changes in either the protein or carbohydrate of the diet affect the yield and composition of milk were investigated. The animals were put on approximately adequate rations. In two experiments the energy was reduced 33 per cent; in one of these the protein also was cut 50 per cent and in the other it was unchanged. In two other experiments the protein was cut 50 per cent; in one the quality of the diet protein was also reduced and in the other it was kept constant. In the fifth experiment only the quality of the diet protein was varied. In the first four experiments the cows were finally put back on the original rations. The amino N of the blood and plasma and the yield and composition of the milk were followed. The results may be summed up as follows:

1. The cut in the amounts of energy or protein or both in the ration reduced the milk yield and the concentration of milk N. The concentration of lactose was unchanged, and that of milk fat was reduced only when the protein alone was reduced. The amino N of the blood and plasma was reduced except when the energy of the ration was unchanged and the quality of the diet protein was also reduced. Reducing the quality of the diet protein either with or without a reduction in quantity reduced the milk yield without reducing the amino N of the blood and plasma.

2. When these changes in diet were reversed the changes in the yield and composition of the milk were reversed, but these changes were not the exact reciprocals of those produced by lowering the rations. Only when the amount of protein was alone increased without any change of quality did the amino N of the blood rise. It was, in general, at first reduced.

The results indicate that changes in either the carbohydrate or protein of the diet affect the yield and composition of milk through

changes in the quality and quantity of the amino-acid mixture of the blood, and that the quality of the diet protein is an important factor in effecting these changes.

ACID-BASE METABOLISM IN INFANTS.

By ALFRED T. SHOHL.

(From the Department of Pediatrics, Yale University School of Medicine, New Haven.)

A new principle in the study of acid-base metabolism is presented. The food and excreta are analyzed for all the acids and all the bases. Their acid or base value is calculated in cc. of normal solutions. From the value of the intake is subtracted the value of the output. The remainder represents the balance. If the body is in equilibrium the balance is zero, for the output equals the intake. In acidosis, if base is excreted, it represents acid retention or abnormal acid production. If there is an acid excretion it represents a retention of base by the body. In infants of 1 year the retention is roughly 100 cc. 0.1 N base per day.

The acid-base value of the urine can also be determined by titration according to Palmer and Henderson. The acid excretion equals the free acid plus ammonia. However, if organic acid is present this must be determined and subtracted. In alkaline urines the carbonates must also be determined and subtracted. For as the organic acids bind base so the carbonates too unite with base and, therefore, represent base excretion. The carbonates, therefore, act as buffers and preserve neutrality in alkaline urines. Unless these corrections are made the acid-base value of the urine cannot be determined by titration.

A method is presented for the titration of the acid-base value of the stools. The principle is to determine the organic acid by extraction. The stool is then titrated in alcoholic solution and the acid-base value determined by subtraction. If carbonates are present they must be subtracted as in the case of the urine. In the stools the buffer value of the phosphates is unimportant. The neutrality is preserved by the fatty acids and soaps and by the carbonates.

RESPIRATORY QUOTIENT STUDIES IN SCURVY AND BERI-BERI.

By H. J. GERSTENBERGER and C. W. BURHANS.

(*From the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland.*)

This study, begun during the close of 1916, was started with the idea of bringing evidence for or against the theory that scurvy was the result of a disturbed carbohydrate metabolism, caused, owing to the absence of or the reduction in the amount of antiscorbutic vitamine in the diet, by the establishment of a disproportion between the quantity of the antiscorbutic vitamine in the food on the one hand and its carbohydrate content on the other. The relative scarcity of scorbutic infants and early difficulties in getting scorbutic guinea pigs to take adequate amounts of food at the desired time was responsible for the inclusion of beri-beri in this study, a disease which according to Funk can be produced with greater rapidity by increasing the carbohydrate intake of the pigeon.

Whenever the opportunity was offered scorbutic infants were studied. Finally, by using Kellers malt soup, it was possible to establish satisfactory experimental conditions regarding scurvy and food intake in guinea pigs and to study the respiratory quotients in these animals.

The results of these studies bring evidence that scorbutic infants and guinea pigs, as well as polyneuritic pigeons can burn carbohydrates completely, and that, therefore, the study of respiratory quotients of animals ill with scurvy or beri-beri brings no evidence in favor of the correctness of the above mentioned theories.

CHEMICAL BLOOD CHANGES IN PNEUMONIA.

By JOHN A. KILLIAN.

(*From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.*)

These studies comprise determinations of the non-protein and urea nitrogen, uric acid, creatinine, sugar, chlorides, and carbon dioxide-combining power of the blood in cases of pneumonia of various types. In some instances, also, parallel estimations of

the total nitrogen and chlorides of the urine have been made. A significant rise in the undetermined nitrogen of the blood has been noted about the period of the resolution of the pneumonia. Consequent to this, an impairment of kidney function develops, resulting in an accumulation of the nitrogenous waste products in the blood. The rise in the non-protein nitrogen is followed by an increase first, in the uric acid; later, in the urea nitrogen; and finally, in the creatinine. In fatal cases a rise of the creatinine to more than 4 mg. per 100 cc. was noted. An attempt has been made to correlate the changes in the chloride concentration and the carbon dioxide-combining power of the blood throughout the course of the pneumonia with the clinical manifestations of the disease.

FURTHER STUDIES ON THE REACTION OF DYING TISSUES.

By WITHROW MORSE and R. GOLDBERG.

(From the Laboratory of Physiological Chemistry, West Virginia University, Morgantown.)

It has been reported by others and by the authors that as tissue dies, the low alkalinity passes to a relatively high acidity. Preliminary experiments showed that this reaction takes place with great rapidity, and Morse and van der Heyde have reported the results of a study of mammalian liver using the gas chain method for estimating acidity. In this study the concentration of hydrogen ions within a few moments after excision of the liver was found to be in the neighborhood of pH 4.6. Criticism had been directed against these results since there must be time given for equilibrium to be reached, and in order to rule out this difficulty we have repeated the experiments, with modified technique, using the Sörensen colorimetric method upon ice-cold alcoholic extracts, following somewhat the technique of Fletcher and Hopkins in their work on lactic acid in muscle. The same average results were obtained in this procedure as we obtained with the electrometric method. Attention was then turned to lactic acid as being responsible for the sudden development of acidity. A basal figure for lactic acid estimated as zinc lactate by the zinc oxide method was found to be 0.035 gm. per cent wet weight of tissue. This figure approaches that obtained by Fletcher

and Hopkins and more recently by Foster and Moyle for basal lactic acid in frog muscle. This concentration of lactic acid is adequate to explain the acid figures. Kidney tissue gives similar acid figures.

CALCIUM METABOLISM IN TETANY.

By FRANK P. UNDERHILL, WILDER TILESTON, and L. JEAN BOGERT.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

When compared to normal individuals under the same experimental conditions a subject with tetany, presumably of gastrointestinal origin, showed a normal type of behavior to calcium intake except that there was evidence of a greater tendency to store calcium temporarily on a calcium-rich diet. On the other hand, on a calcium-poor diet this stored calcium is eliminated to a much greater extent than occurs in the normal subject. These facts may, perhaps, be interpreted to mean that the organism with tetany shows a greater need for calcium than the normal individual but that in tetany the regulation of calcium equilibrium is in an unstable condition.

INDOLETHYLAMINE IN THE URINE OF PELLAGRINS.

By M. X. SULLIVAN.

(From the Hygienic Laboratory, Washington.)

About 40 liters of mixed urine of pellagrins were treated with lead acetate and with basic lead acetate and the filtrate, freed from lead, was concentrated to 1,500 cc. The concentrated solution, made 5 per cent acid with H_2SO_4, was treated with phosphotungstic acid in 5 per cent H_2SO_4. In the filtrate from the resulting precipitate, indolethylamine was found. The solution, freed from phosphotungstic acid by baryta and from barium by dilute H_2SO_4, was concentrated *in vacuo* to 300 cc. and filtered. The filtrate was strongly acidified with concentrated HCl and filtered from a precipitate which formed. The filtrate, concentrated to 100 cc., was treated with 95 per cent alcohol and

filtered. The filtrate, freed from alcohol, was treated with picric acid. A dark red precipitate of long needles occurred which, on recrystallization from acetone, had a melting point of 242°C. From the picrate a chloride was obtained, melting at 244–245°. A small amount of free base was likewise obtained of which the melting point was not obtained. The free base and syrup, however, gave an intense violet-blue color with glyoxylic and sulfuric acids as did the hydrochloride, likewise. From the color, crystalline shape, solubility, and melting point of the picrate, the melting point and the Hopkins and Cole's reaction of the chloride, and the Hopkins and Cole's reaction of the free base, it is evident that the compound is indolethylamine.

THE CHICK AS AN EXPERIMENTAL ANIMAL IN VITAMINE STUDIES.

A PRELIMINARY REPORT.

By A. D. EMMETT and GAIL E. PEACOCK.

(From the Biological and Research Department, Parke, Davis and Company, Detroit.)

Four series of feeding trials were carried out using young White Leghorn chicks in comparison with rats and pigeons. It was found that in modifying the rations for the chicks by increasing the nitrogen, introducing some roughage, and supplying a liberal amount of charcoal, oyster shell, and grit, that the birds were seemingly well adapted to studies where either the vitamine A or B was absent.

The chicks were all fed for the first 2 weeks after hatching in the most approved practical manner so as to tide them over the initial critical period of growth. They were then placed in confinement and fed on the same ration for 7 to 10 days when they were given the various experimental diets. The chicks fed upon the normal control diet grew and gained at the usual rate and had the appearance of healthy fowl. Those fed upon the vitamine B minus diet soon showed definite signs of the deficiency—ceasing to grow, losing in weight, and developing other symptoms of malnutrition, culminating in polyneuritis in 10 to 12 days. Hydropericardium, hypertrophy of the adrenals and gall bladder, and atrophy of testes were found on autopsy. The

chicks fed the vitamine A minus ration continued to gain normally for a longer time than did those on a lack of the vitamine B. They showed a weakness of legs; poor condition of the feathers; fading or bleaching of the pigment in the legs, bill, and toes; anemia; edema about the eye, followed by an ophthalmic condition similating xerophthalmia; beading of the ribs; atrophy of the testes, and hypertrophy of the adrenals and gall bladder. The period of duration was from 14 to 21 days. In the case of the vitamine C, the requirements of the chick appear to be much less than for vitamines A and B.

The advantages in favor of the chicks are: ease of obtaining large numbers of animals; the minimum amount of attention and lessened cost compared with the breeding of the rat; the greater uniformity of the animals and hence lessened degree of variation; and a shortening of the time and duration of the feeding tests, due to the apparent increased sensitiveness of the chick to the lack of the vitamines.

The possibilities of the chick in such feeding trials appear to suggest that they are both promising and practical.

CALCIUM IN EGG-SHELL FORMATION.

By G. D. BUCKNER, J. H. MARTIN, W. C. PIERCE, and A. M. PETER.

(From the Kentucky Agricultural Experiment Station, Lexington.)

Six lots of 7 month old White Leghorn pullets were fed for 8 months, restricted as follows:

Lot 1.	Grains	+	tankage	+	no mineral material.	
“ 2.	“	+	“	+	granite grit, *ad libitum*.	
“ 3.	“	+	“	+	“ “ “ “	+ oyster shell, *ad libitum*.
“ 4.	“	+	“	+	“ “ “ “	+ limestone “ “
“ 5.	“	+	“	+	limestone, “ “	
“ 6.	“	+	“	+	rock phosphate, *ad libitum*.	

Tankage (12.5 per cent of total feed) containing 6.4 per cent P_2O_5 was fed in the mash. The grit used contained 2.42 per cent CaO soluble in strong HCl.

Results obtained from the analyses of the leg bones and the egg-shells and the number of eggs produced in each lot indicate

that the calcium carbonate can be utilized by the hen for the production of egg-shells and bones but that the calcium in tricalcium phosphate can only be utilized for the growth of bones and not for egg-shell production. Also that calcium starvation is not the determining factor in the production of shell-less eggs.

THE HEAT OF ENZYME REACTION.

A STUDY OF THE HEAT PRODUCED IN THE CATALASE REACTION.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

In a previous paper on the catalase reaction[10] as well as in the publications of earlier investigators the fact has been noted that the velocity constant of the reaction in the first few minutes diminishes very rapidly. It seemed probable that these changes are due to temperature alterations, and the experiments here reported were performed to test this hypothesis. The results demonstrate that the catalase reaction is exothermic, and that it is accompanied by a definite heat production.

The experiments were made with a preparation of liver catalase. The volume of the mixture and its reaction (pH = 7) were kept constant in all tests. Only the relative amounts of catalase and hydrogen peroxide varied. The reaction took place in a thermos bottle, and the rise in temperature was measured with a special thermometer. Experiments with boiled solutions of catalase showed that only a negligible heat production (0.02°C. per minute of shaking) occurs which must be used as a correction in the experiments with the unboiled catalase. Without discussing the weak points of the technique, the heat production in the catalase reaction is now an established fact, as is shown by the data recorded below. The curves of heat evolved differ in no essential respect from curves plotted from data of oxygen set free in the reaction. The maximum heat production occurs in the first 5 minutes; *i.e.*, during the period when the velocity

[10] Morgulis, S., *J. Biol. Chem.*, 1921, xlvii, 341.

constant changes most abruptly. It is, therefore, concluded that the abnormalities in the reaction velocity are directly associated with the thermal phenomena accompanying the catalase reaction. The heat production is evidently conditioned upon the

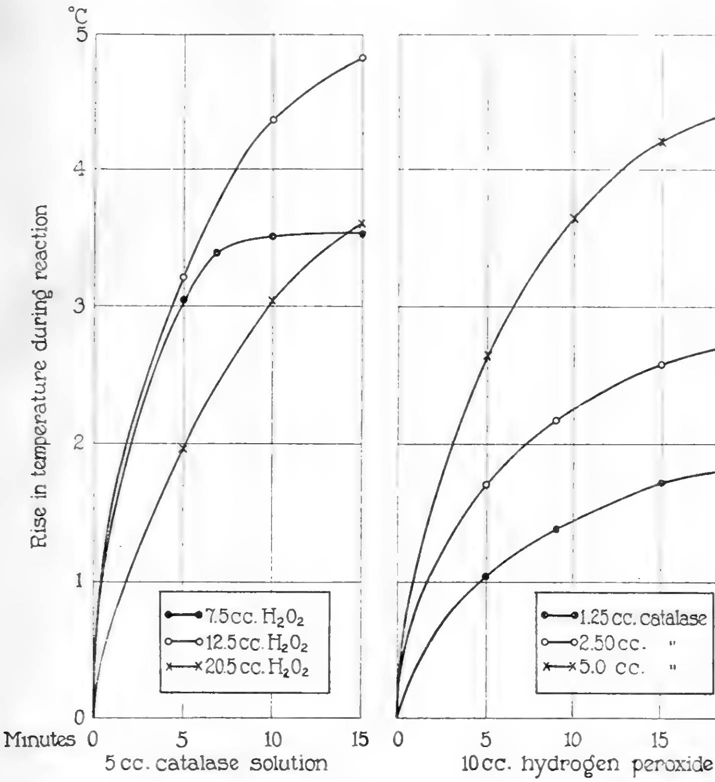

Fig. 1.

intensity of the reaction, and by further refinement of the apparatus it is expected that it will be possible to follow the progress of the reaction by the temperature changes not only for catalase but for other enzymes as well.

FURTHER EXPERIMENTS ON THE PREVENTION OF RICKETS IN RATS BY EXPOSURE TO LIGHT.

By ALFRED F. HESS.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.)

The development of rickets in rats fed the rachitic diet described by Sherman and Pappenheimer, can be prevented by daily exposures to direct sunlight for 15 minutes. Sunlight which has traversed flint window glass loses this potency. After having been reflected from a white surface it retains some of its effectiveness. Rickets, likewise, can be prevented by exposure for about 2 minutes to the rays from a mercury-vapor lamp. The ozone emanations from this lamp have no protective value. Rays from an open carbon arc lamp protect. Soft x-rays are ineffective.

Prolonged exposure to direct sunlight failed to prevent or to delay the onset of scurvy in guinea pigs.

GROWTH AND REPRODUCTION IN RATS ON A MILK DIET.

By H. A. MATTILL.

(From the Department of Physiology, University of Rochester, Rochester.)

The failure of adolescent growth and of reproductive ability in female rats on whole milk powder was not corrected by diluting the milk powder with lard, starch, and salts in varying proportions. The animals still showed the characteristic slowing of growth rate at adolescence and were infertile. Males grew normally but their reproductive efficiency was questionable. Additions of protein-free milk, of cod liver oil, or of traces of KI after the animals had reached the age of 150 to 175 days did not result in fertility or renewed growth. Ordinary stock rat food did not do it. When animals that grew up on stock food were transferred to milk rations at about the age of adolescence they likewise failed to rear their young, or were sterile.

The addition of a small amount of yeast to milk rations caused the females to cast litters regularly and repeatedly, but none ever lived more than a few days.

Satisfactory correlation between fertility and weight of gonads is not yet possible because the variability is too large for the number of animals thus far observed.

Of 16 males on milk rations	8 had average testes weights varying + 16 per cent from normal and
	8 had average testes weights varying − 42 per cent from normal.
Of 31 females on milk rations	6 had average ovary weights varying + 16 per cent from normal and
	25 had average ovary weights varying − 36 per cent from normal.

The plus figures are undoubtedly within the range of normal variability; the minus figures may not be. The partial success induced by yeast addition did not materially alter this distribution. Underdeveloped testes seldom, if ever, contained spermatozoa and histological material is now being studied.

RIGOR MORTIS.

By J. B. COLLIP.

(From the Department of Pathological Chemistry, University of Toronto, Toronto, Canada.)

When muscle plasma is carefully acidified, a precipitate of the proteins is brought about. This precipitate redissolves on the further addition of acid and reappears when alkali is slowly added, and again redissolves on the further addition of alkali. If one filters at the point of maximal precipitation the pH of the filtrate falls between 6.3 to 6.6 index. This corresponds to the pH index of muscle going into rigor. It is, therefore, suggested that the onset of rigor is due to physical changes, the development of postmortem acidity in the tissue resulting in rigor when the isoelectric point of the proteins is reached. The passing of rigor may be associated both with the development of further acidity and with enzymatic action.

STUDIES OF THE NORTH AMERICAN SARRACENIACEÆ.

By JOSEPH S. HEPBURN, E. QUINTARD ST. JOHN, FRANK M. JONES, and WILLIAM F. BAKER.

(From the Constantine Hering Laboratory, Hahnemann Medical College, Philadelphia.)

Liquor from closed pitchers of *Darlingtonia californica* contained diastase; protease, maltase, emulsin, invertase, and urease were absent. Liquor from closed pitchers of *Sarracenia flava* contained invertase and lipase; maltase, emulsin, diastase, urease, and esterase were absent; it had a surface tension of 66.4 dynes per cm. Protease occurred in the liquor from closed pitchers of the *Sarraceniæ*. Liquor from closed pitchers was bacterially sterile, that from open pitchers contained proteolytic bacteria. The pitchers absorbed nutrient compounds from their cavities. The pitcher liquor of the *Sarraceniæ* produced permanent cessation of motion and caused ants to sink more frequently and more promptly than in water. The liquor did not hemolyze human erythrocytes; that from closed pitchers had no toxic action when injected into the lymph sac of frogs or subcutaneously into a guinea pig. Prolonged administration of the mother tincture of either the rhizomes or the pitchers of *Sarracenia minor*, *Sarracenia flava*, *Sarracenia drummondii*, or *Sarracenia rubra* to rabbits by mouth in small doses produced allopecia, formation of red punctate umbilicated papules, rupture of the postules, formation of crusts, and other symptoms. The rabbits recovered when medication ceased, and had then acquired an immunity to the action of the drug. The rhizomes did not contain protease.

CHANGES IN THE REFRACTIVE INDEX OF THE BLOOD SERUM OF THE ALBINO RAT WITH TEMPERATURE.

By F. S. HAMMETT and IDA S. TELLER.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

A study of the changes in the refractive index of the blood serum of the albino rat with rising temperature showed that two types can be differentiated according to the nature of the response. In the first type the changes in the refractive index coincide with

those of the solvent water and can be attributed to this serum constituent. In the second type, the curve of the changes of refraction with rising temperature falls away from that of water. This demonstrates a participation in the response of serum constituents other than that of the solvent water. The factors which contribute to this difference are unknown, although there is a possibility that a seasonal variation may be a determinant. It is certain that in this series the factors of body length, body weight, age, water content of serum both before and after the experiments, and previous state of digestion and absorption are not the causes of the difference between the two groups.

The correction for the reduction of the observed angle of refraction to the common base at 20° when readings are taken at different temperatures is obtained by the use of the formulas;

$$I = i - 1.25' \ (t-20) \text{ and}$$
$$I = i + 1.25' \ (20-t)$$

where I is the corrected angle of refraction; i, the observed angle of refraction; t, the observed temperature; and 1.25′, the change in minutes for each degree of change in temperature. These formulas hold for temperatures between 17.5 and 35°C.

TOTAL METABOLISM IN EXOPHTHALMIC GOITER.

By WALTER M. BOOTHBY AND IRENE SANDIFORD.

(From the Section of Clinical Metabolism, Mayo Clinic and the Mayo Foundation, University of Minnesota, Rochester.)

The work included a quantitative study of the food intake, urinary elimination, the blood chemistry, and the respiratory metabolism in three cases of exophthalmic goiter. The total metabolism was found to be frequently in excess of 5,000 calories per day and occasionally over 6,000 calories, which is in marked contrast to the daily food ration of 1,500 to 1,800 calories common in many countries during the war.

THE METABOLISM OF INORGANIC SALTS.

By ERWIN G. GROSS and FRANK P. UNDERHILL.

(From the Department of Pharmacology and Toxicology, Yale University, New Haven.)

In normal dogs the relationships existing between calcium, magnesium, potassium, sodium, etc., in the blood are remarkably constant under fixed experimental conditions. When changes occur in one direction an immediate compensation occurs.

THE APPARENT ACID DISSOCIATION CONSTANTS OF OXYHEMOGLOBIN AND REDUCED HEMOGLOBIN.

By EDWARD A. DOISY, A. P. BRIGGS, and K. S. CHOUKE.

(From the Laboratories of Biological Chemistry, Washington University, School of Medicine, St. Louis.)

Determinations were made of the distribution of base between oxyhemoglobin or reduced hemoglobin and carbonic acid. From these data, the apparent dissociation constants of the two forms of hemoglobin were calculated.

The isoelectric points of oxyhemoglobin and reduced hemoglobin were determined by equilibrating the hemoglobin-base solutions with high tensions of carbon dioxide. The point at which the combined carbon dioxide equals the inorganic base of the solution was taken as the isoelectric point.

By plotting the bicarbonate values against pH, the relationship of loss of oxygen to increase of bicarbonate at a constant pH was obtained. The magnitude of these values was of the same order as those obtained on defibrinated blood. The loss of approximately 2 volumes of oxygen allows the blood to take up 1 volume of carbon dioxide without a change of hydrogen ion concentration.

COLORIMETRIC METHODS FOR THE DETERMINATION OF HOMOGENTISIC ACID AND MAGNESIUM.

By A. P. BRIGGS.

(From the Laboratories of Biological Chemistry, Washington University School of Medicine, St. Louis.)

Phosphomolybdic acid is especially susceptible to reduction by *p*-diphenols. Advantage of this reaction has been taken, first, to determine homogentisic acid in alcapton urine, and again to determine magnesium by the phosphorus content of the $MgNH_4PO_4$ precipitate.

ON THE RELATION OF THE HYDROGEN ION CONCENTRATION TO THE FERTILIZATION OF MARINE EGGS.

By G. H. A. CLOWES and HOMER W. SMITH.

(From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.)

The permeability of sea urchin and starfish eggs to homologous sperm varies with the hydrogen ion concentration of the sea water, regardless of whether the acid involved is carbonic acid or a mineral acid. In each species there is a hydrogen ion concentration at which the permeability to sperm is greatest, and another at which the permeability is zero. This indifference to the nature of the acid employed exhibits a striking contrast to the results on cell division reported in a preceding paper.[11]

ON PHYSICAL AND CHEMICAL VARIATIONS IN THE COMPARISON OF INTERIOR AND SURFACE PROTOPLASMA.

By G. H. A. CLOWES, ROBERT L. CHAMBERS, and IRVINE PAGE.

(From the Biochemical Research Laboratories, Eli Lilly and Company, Indianapolis.)

By comparing the relative cytolytic effects exerted by saponin and digitonin on the one hand, and hypotonic solutions on the other, it was possible to demonstrate that the surface of marine eggs contains a larger proportion of cholesterol than does the interior, or cholesterol is present in a more effective form as a protective agent against the action of saponin, digitonin, etc.

SOURCES OF ERROR IN THE DETERMINATION OF CHLORIDES IN BLOOD.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

In the determination of chlorides in blood, it is usual to precipitate the protein by some acid precipitant. It is generally assumed that the precipitating anion completely displaces the chlorine ion from combination with protein. This is not always the case, particularly when nitric acid or metaphosphoric acid is used.

If picric acid is used as the precipitant, the presence of a purine, apparently hypoxanthine, in the cells makes the results too high

[11] Clowes, G. H. A., and Smith, H. W., *J. Biol. Chem.*, 1922, l, p. iv.

unless about 20 per cent nitric acid is present. The precipitate of hypoxanthine—silver-picrate—contains much more silver than the molecular formula given in the literature would require.

A MICRO METHOD FOR THE DETERMINATION OF UREA IN BLOOD.

By ISRAEL S. KLEINER.

(From the Department of Physiological Chemistry of the New York Homeopathic Medical College and Flower Hospital, New York.)

The method consists in direct Nesslerization of a Folin-Wu filtrate after the urea has been digested by urease. The color is compared, in the Klett-Kleiner micro colorimeter, with a 1 per cent potassium bichromate solution in a test-tube wedge mounted on a deep yellow glass background. The reading is made and the percentage of urea found by consulting a table.

ANALYSIS AND COMPOSITION OF CORN POLLEN.

By R. J. ANDERSON and W. L. KULP.

(From the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.)

Having failed to find any reference in the extensive literature dealing with corn and corn products regarding the composition of corn pollen, we have undertaken an investigation of this substance. Since pollen plays an all important part in the process of fertilization and reproduction, it would seem as if some knowledge regarding the chemical compounds occurring in pollen would be of interest to plant physiologists.

Our results indicate that different varieties of corn produce pollen which varies greatly in composition and this fact might be of importance in cross-breeding.

The approximate composition of the pollen from three varieties of corn has been determined and a complete analysis of the ash of the pollen from one variety of corn has been made. Evidence is presented which indicates the presence of at least two phosphatides in corn pollen—one was an amorphous substance which also contained sulfur and the other was crystalline, corresponding in composition to a diaminomonophosphatide. Relatively large quantities of free inosite, *l*-proline, and choline were also isolated.

A COMPARATIVE STUDY OF THE COMPOSITION OF THE FEMUR.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

A few years ago, through the courtesy of Dr. S. J. Foote, I came into possession of an interesting collection of femurs. In view of the limited number of comparative determinations of bone composition, this material was examined chemically. Without entering here into a discussion of the analytical methods, it may be stated that the bones were cautiously freed from all traces of adhering tissue, dried, and ether extracted. The analyses were, therefore, made on water-free and fat-free material. The present report does not include the data for femurs of Egyptian Mummy or Pueblo Indian. The analyses extended to a determination of organic substance, ash, carbon dioxide, calcium, magnesium, and phosphorus. The quantitative data were calculated as calcium carbonate, magnesium phosphate, and calcium phosphate. Recognizing certain objections to this method of expressing the composition of the bone, it is, however, preferable to the conventional way of giving it in terms of percentages of Ca, Mg, P, and CO_2.

The composition of the bones examined reveals a remarkable uniformity. The percentage of CO_2 in the ash varies from 4.33 to 5.68, that of the Ca from 37.58 to 38.70, of Mg from 0.57 to 0.86, and that of P from 18.03 to 18.90 per cent. The femur of the turtle is an exception in that it contains a much greater proportion of CO_2 (8.43 per cent) and a lower percentage of phosphorus (17.02 per cent) than the other bones studied. Incidentally, it is also the softest bone with the largest content of organic matter.

Hoppe-Seyler regarded the salts of bone tissue as being similar to the mineral apatite $Ca_{10}\ Fl_2\ (PO_4)_6$ in which CO_3 takes the place of Fl_2. In a salt of this composition the Ca as phosphate and as carbonate should be in a ratio of 9.3:1 (930.87:100.08). The last column of the table gives these ratios as determined from our analytical data; the ratio will be seen to vary greatly, from 3.62 in the case of the turtle to 9.11 in the human femur. The ratios for the other femurs examined range all the way between these extremes. The lack of constancy in the ratio between the

calcium phosphate and calcium carbonate seems to invalidate Hoppe-Seyler's assumption.

Femur from:	Composition of bone.				Composition of ash.			
	Organic matter.	$CaCO_3$	$Mg_3(PO_4)_2$	$Ca_3(PO_4)_2$	$CaCO_3$	$Mg_3(PO_4)_2$	$Ca_3(PO_4)_2$	$\frac{Ca_3(PO_4)_2}{CaCO_3}$
Elk	29.40	7.20	1.59	62.50	10.19	2.55	88.50	8.68
Sheep	29.56	7.76	2.18	60.40	10.92	3.10	85.60	7.84
Mule	30.15	8.03	2.07	61.05	11.49	2.96	87.30	7.60
Hippopotamus	30.26	8.09	1.86	60.90	11.60	2.66	87.40	7.09
Turkey	30.51	7.48	1.86	61.45	10.76	2.67	88.30	8.42
Frog	32.36	8.45	1.66	58.35	12.50	2.45	86.30	6.89
Dog (bull)	32.98	7.42	1.58	57.50	11.08	2.35	85.80	7.98
Man	33.19	6.59	1.40	60.00	9.86	2.09	89.70	9.11
Horse	33.82	8.56	1.36	52.00	12.93	2.06	86.10	6.66
Turtle	37.23	12.03	1.95	49.85	19.16	3.10	79.40	3.62

A STUDY OF THE NON-PROTEIN CONSTITUENTS IN BLOOD OF SOME MARINE INVERTEBRATES.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha.)

The non-protein components of blood have proved themselves an invaluable aid in the investigation of metabolism of the higher animals. This consideration led the author to undertake a survey of the blood of invertebrates. The present investigation is confined to arthropods from which sufficient blood for analysis can easily be obtained.[12]

The splendid system of blood analysis outlined by Folin and Wu has been followed in this research with some minor modifications necessitated by the nature of the material. This will be fully discussed in the detailed report. The blood from *Limulus*, blue crab, spider crab, and lobster was examined and the results tabulated below.

[12] It is a pleasure to acknowledge the help received from Dr. Leo Loeb in procuring the bloods.

Non-Protein Constituents of the Blood of Arthropods.

Animal.	Mg. per 100 cc. of blood.			Remarks.
	Sugar.	Non-protein nitrogen.	Uric acid.	
Lobster (*Homarus*).	26	13.0	2.0	
	20	12.8	2.5	
	19	12.5	2.1	
	20	13.3	2.4	
Spider crab (Labinia).	45	33.0	1.0	Fresh batch.
	25	22.0	2.3	" "
	27	23.0	2.3	" "
	29	19.5	2.3	" "
	26	16.0	3.8	?
	43	13.0	2.0	Several days old.
	43	15.0	1.0	" " "
Blue crab (Callinectes).	182	24.7	2.9	Fresh from the sea.
	64.5	22.7	3.4	" " " "
	41.5	8.0	0.3	Used on the day of delivery to laboratory.
	17.5	13.6	0.4	
	9.5	9.3	Negative.	1 day in aquarium.
	18.0	18.6	"	
	12.5	9.0	"	2 days in aquarium.
	13.8	10.0	"	
Limulus.	34.0	26.0	0.3	Recently caught.
	22.5	20.0		1 day in tank.
	12.5	14.0	0.7	2 days in tank.
	5.0	10.0		After many weeks in confinement.
	7.5	13.0	0.8	

The findings may be summed up as follows: the sugar, non-protein nitrogen, and uric acid content of the blood shows very great variability, except in the lobsters. The greatest variability occurs in bloods from blue crabs, the smallest in that of the lobster. The uric acid is present in large amount in both lobster and spider crab blood, in blue crabs only in specimens examined almost as soon as they have been removed from the water. The content of the non-protein materials of the blood evidently de-

pends on the condition of the animals (nutritive?). This is especially well shown in the blue crab which is an exceedingly active animal. Examined immediately upon being brought to the laboratory they showed a variable but high content of the non-protein substances, but after having been kept in the aquarium for 1 day the sugar and non-protein nitrogen diminish to practically the lowest level, while the uric acid has disappeared completely. The condition in *Limulus* is similar, and in the spider crab the same state of affairs exists though perhaps not quite so definitely expressed. On the contrary, the blood from lobsters shows very distinctly a stable composition of the non-protein components. The results suggest that in these arthropods different stages in the development of the mechanism for the regulation of the blood composition are represented. The influence of the nutritive state upon the blood composition, and its relation to the degree of development of the excretory mechanism will be the subject of direct experimentation.

THE REACTIVITY OF THE MOLYBDENUM AND TUNGSTEN REAGENTS OF FOLIN.

By VICTOR E. LEVINE and BERNARD C. BURNS.

(*From the Biochemical Laboratory, School of Medicine, Creighton University, Omaha.*)

To test out the reactivity of the Folin reagents 225 compounds of biochemical interest were employed. About 100 compounds responded to the phenol reagent of Folin and Denis. Among these were amino-acids, proteins, aldehydes, ketones, carbohydrates, and alkaloids. Creatinine was positive; creatine, negative. Most compounds reacted at room temperature while others required heating (formic acid, glucose, caffeine, etc.). In view of these findings the phenol reagent is worthless in the detection of the antineuritic vitamine, and the quantitative methods for phenol, cresol, and tyrosine must be modified so as to remove interfering substances from the compound under estimation. This criticism also applies to the estimation of adrenalin with the uric acid reagent.

The introduction of nitro groups in phenol renders the reagent

inactive. Mono-, *o*-, and dinitro- and trinitrophenol (picric acid) do not respond. Picraminic acid, however, is positive.

The uric acid reagent proved less reactive, only 45 compounds yielding positive results. Creatinine is positive; and creatine, negative. The estimation of uric acid does not suffer in accuracy from the non-specificity of the reagent, since in the method the uric acid is separated by precipitation from interfering substances.

In the system of blood analysis of Folin and Wu[13] there is recommended in the determination of sugar a reagent reacting with cuprous oxide. It is interesting to find that it reacts with inorganic reducers (cuprous oxide or chloride, ferrous sulfate, sulfites, sulfides, stannous chloride, etc.) and with only two organic compounds, which contain the hydrazine grouping—hydrazine and phenylhydrazine.

THE ACID-BASE EQUILIBRIUM IN THE BLOOD AFTER PARATHYROIDECTOMY.

By D. WRIGHT WILSON and C. I. KRANTZ.

A METHOD FOR THE DETERMINATION OF URIC ACID.

By STANLEY R. BENEDICT.[14]

SOME PHYSIOLOGICAL AND CHEMICAL PROPERTIES OF THYROXIN.

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NORMAL SUGAR EXCRETION IN RELATION TO CARBOHYDRATE INTAKE AND BLOOD SUGAR FLUCTUATIONS.[15]

By OTTO FOLIN and HILDING BERGLUND.

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[13] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

[14] Benedict, S. R., *J. Biol. Chem.*, 1922, li, in press.

[15] Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, in press.

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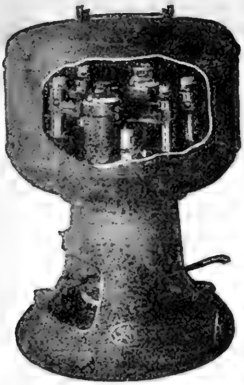

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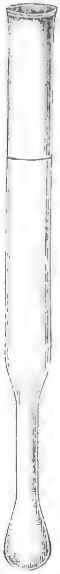

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